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<b>(57) Abstract</b> <p>Methods, assays, and components are described in which biological samples can be rapidly and sensitively analyzed for the presence of species associated with neurodegenerative disease. Techniques and components are provided for diagnosis of disease, as well as for screening of candidate drugs for treatment of neurodegenerative disease. The techniques are simple, extremely sensitive, and utilize readily-available components. Binding species, capable of binding a neurodegenerative disease aggregate-forming or fibril-forming species, are fastened to surfaces of electrodes and surfaces of particles, or provided free in solution, to bind fibril-forming species and/or be involved in aggregation.</p>			

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(54) Title: **RAPID AND SENSITIVE DETECTION OF ABERRANT PROTEIN AGGREGATION IN NEURODEGENERATIVE DISEASES**

(57) Abstract: Methods, assays, and components are described in which biological samples can be rapidly and sensitively analyzed for the presence of species associated with neurodegenerative disease. Techniques and components are provided for diagnosis of disease, as well as for screening of candidate drugs for treatment of neurodegenerative disease. The techniques are simple, extremely sensitive, and utilize readily-available components. Binding species, capable of binding a neurodegenerative disease aggregate-forming or fibril-forming species, are fastened to surfaces of electrodes and surfaces of particles, or provided free in solution, to bind fibril-forming species and/or be involved in aggregation.

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## RAPID AND SENSITIVE DETECTION OF ABERRANT PROTEIN AGGREGATION IN NEURODEGENERATIVE DISEASES

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### Field of the Invention

This invention relates to methods, assays, and components for the rapid and sensitive detection and analysis of peptide aggregation associated with neurodegenerative diseases. Such methods and assays can be employed for clinical testing as well as to facilitate drug discovery by high-throughput drug screening.

### Background of the Invention

Many neurodegenerative diseases have now been shown to be linked to and/or caused by plaque associated with aggregate and/or fibril formation in the brain that occurs as a result of aberrant aggregation of neuro peptides. Neurodegenerative diseases including Alzheimer's, Parkinson's, Gertsman-Straussler-Scheinker Syndrome, Fatal Familial Insomnia, Huntington's Chorea, Kuru, and Familial amyloid polyneuropathy and transmissible spongiform encephalopathies such as Creutzfeldt Jakob, Scrapie, and Bovine Spongiform Encephalopathy (BSE, Mad Cow), are characterized by ordered protein fibrils, aggregates that form in the brain. Although the proteins that make up these fibrils share no sequence homology, or even conserved motif, the fibrils themselves share certain morphological features. *See* Lansbury, *Proc. Natl Acad. Sci. USA*, 96:3342 (1999). In these diseases, peptides related to the pathogenic state, which are normally soluble, undergo a conversion of their 3-dimensional structure via a mutation of the native peptide or a physical association with altered peptides, to an insoluble, ordered polymerized state that is characteristic of the abnormal protein deposits that are found in the brain in neurodegenerative diseases. This ordered polymerizing or aggregation may result from seeding with indigenous or exogenous agents or peptides.

For example, in Alzheimer's disease (AD), these fibrils are made up of  $\beta$ -amyloid protein that has undergone a conformational change, from soluble monomers, to insoluble,  $\beta$ -sheet oligomers. The concentration of these fibrils in the brain has been correlated to the progression of clinical disease. The growth profile of the characteristic fibrils is extremely non-linear, which



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could explain why its victims can appear asymptomatic for years, then suddenly undergo a rapid degeneration into dementia. Fibril formation *in vitro* is peptide concentration dependent. Short synthetic peptides, derived from the  $\beta$ -amyloid ( $A\beta$ ) protein, can be made to form fibrils *in vitro* to mimic fibril formation that is characteristic of Alzheimer's disease.  $A\beta$  1-42 (with an  
5 extended hydrophobic C-terminus) has been shown to form fibrils at a faster rate than  $A\beta$  1-40. See J. Jarrett *et al*, "The carboxy terminus of the  $\beta$ -amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease," *Biochemistry* 32: 4693-4697 (1993). While  $A\beta$  1-40 can form fibrils or aggregates on its own, solutions  
10 containing  $A\beta$  1-40 undergo accelerated fibril formation if they are "mixed" with the less soluble 1-42 peptide or are "seeded" with pre-formed peptide fibrils. Although  $A\beta$  1-40 is the predominant protein in neuritic plaque, these studies indicate that the rate of fibril formation may depend on the ratio of the concentration of 1-42 to 1-40. Consistent with these findings, all forms of early onset AD involve higher expression levels of the 1-42 peptide.

Atomic force microscopy (AFM) studies of *in vitro* fibril formation using the two most  
15 prevalent variants of  $A\beta$ , 1-42 and 1-40, have identified a meta-stable intermediate; termed the protofibril that occurs before fibril formation. See J. Harper *et al*, "Observation of metastable  $A\beta$  protofibrils by atomic force microscopy," *Chem. and Biol* 4: 119-125 (1997). The existence of these precursors to fibrils may explain the diffuse amyloid deposition observed in the brains of people pre-disposed to AD. The existence of a protofibril that is a toxic intermediate would  
20 explain several observed inconsistencies *in vitro* and *in vivo* that argued both for and against the  $A\beta$  fibril being the pathogenic agent of AD. Existing assays to test for neurodegenerative disease fibrils or fibril-forming species, or to screen for drugs suitable for treatment of neurodegenerative disease are Congo red and Thioflavin-T assays (Methods in Enzymology, Academic Press, 1999, Vol. 309, pgs. 274-287; 304-305) which typically cannot detect small  
25 aggregates or protofibrils. Specifically, they cannot detect aggregate or fibril-forming species at a concentration below about 100  $\mu$ M. This is inadequate for detecting early stage disease, and for screening drugs suitable for use at early stage disease.

Drug candidates that act at a pre-symptomatic stage of the disease (when only small  
fibrils or aggregates are present) would have a greater efficiency in inhibiting plaque/fibril  
30 formation and preventing symptomatic disease. In order to do this, small fibril aggregates need

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to be efficiently detected. However, these aggregates are too small to be detected by nearly every detection method. Although they can be detected by AFM, this technique does not lend itself to clinical diagnostics or drug screening protocols. Therefore, at present, it has not been possible to screen for drugs that would act on the smaller fibrillar species. Additionally, screening for drugs to inhibit fibril formation at any stage has been severely limited.

The rate of aggregate and fibril formation is an extremely non-linear function of the concentration of converted or misfolded peptide, such as mutant neuro peptide or converted prion peptides, which are aggregate-forming or fibril-forming species. Once the concentration of the aberrant species reaches a critical concentration, the reaction rate proceeds too quickly to be affected by drug treatment. Therefore, for a drug to be effective at inhibiting plaque formation, thus making it a preventative therapeutic rather than a palliative one, it would necessarily have to act at an early stage. Current state of the art technology is not capable of detecting small aggregates or fibrils in a manner that is compatible with parallel drug screening methods or non-invasive diagnosis. This means that: 1) drugs to treat the early disease state cannot be readily identified; 2) pre-symptomatic patients cannot be identified; and 3) the effectiveness of potential drug candidates that exist, or will be identified in the future, cannot be accurately assessed.

Another drawback of existing technologies, for example, Congo Red and Thioflavin-T is that they require mechanical intervention during the process. That is, the process requires transfer of fluid from one container to another, and the like which disturbs the assay in a non-reproducible way accelerating fibril formation. Additionally, addition of the Congo Red and Thioflavin components quenches the reaction and stops the aggregation process, thus several time points cannot be taken of a single assay in solution.

One complication with the diagnosis of neurodegenerative disease is the fact that species capable of forming aggregates or fibrils characteristic of the disease may be present in extremely low concentration, yet at a concentration which, if detectable, could signify onset of the disease.

While information exists as to the process of neurodegenerative disease, specifically with regard to diagnoses and drugs screening, simple, inexpensive, and reliable assays for neurodegenerative disease, techniques for screening candidate drugs for treatment of neurodegenerative disease, and related components would be a significant advance in the field.

### Summary Of The Invention

The present invention provides a series of compositions, articles, kits, and methods associated with neurodegenerative disease. Techniques and components are provided for diagnosis of disease, as well as for screening of candidate drugs for treatment of neurodegenerative disease. The techniques are simple, extremely sensitive, and utilize readily-available components. The techniques and components of the invention are able to very sensitively detect neurodegenerative disease aggregate-forming or fibril-forming species, which typically are very small, e.g., between 1 and 100nm in length and have heretofore been difficult or impossible to detect with existing technology and in screening for drugs to treat neurodegenerative diseases.

The present invention therefore contemplates the ability to detect and monitor fibrils or pre-fibrils that are characteristic of early stages of these neurodegenerative diseases. The present invention also contemplates the ability to study the rate of aggregate formation in response to treatment with putative drug candidates.

The present invention provides methods for the detection of aggregates and fibrils which can be used to screen for peptide sequences that are involved in the phenotype of neurodegenerative diseases. In one technique, selected peptide sequences are synthesized and are fastened to or adapted to be fastened to particles and subjected to assays of the invention to see whether particle agglomeration occurs or other binding involving particles as described herein indicative of the peptide sequence's potential for participating in neurodegenerative disease processes. In another technique, random peptide sequences are genetically engineered into a phage, which can be bound to a particle and participate in an assay of the invention. The methods of the present invention provide for detection of fibrils and peptide aggregates and can be used for the diagnosis of these diseases, since it has been shown that fibrillar species circulate in the cerebro spinal fluid (CSF) and other bodily fluids that can be readily sampled.

In one embodiment, the present invention contemplates a detection system in which biological samples can be rapidly and sensitively analyzed for the presence of aggregates, fibrils, and pre-fibrils (aggregate-forming or fibril-forming species) that are characteristic of neurodegenerative diseases, including transmissible spongiform encephalopathis. In one

aspect, the invention provides a series of kits that can be used for disease detection or drug screening. In one embodiment, a kit includes a first article having a surface, a second article having a surface, and a plurality of binding species, at least some of which are fastened to or adapted to be fastened to the surface of the first article, and at least some of which are fastened to or adapted to be fastened to the surface of the second article. The binding species are capable of binding a neurodegenerative disease aggregate-forming or fibril-forming species. As will become apparent from the detailed description below, a variety of combinations of articles can be used in this embodiment. Each article can be a fluid-suspendable, isolatable article, each being a colloid particle or one being a colloid particle and the other being a magnetic bead or the like, or one article can be a surface of a larger article such as an electrode, a surface plasmon resonance (SPR) chip, or other macroscopic article, and the other article can be a fluid-suspendable particle as described above.

Another kit of the invention includes an article having a surface (a variety of articles are contemplated), and a plurality of binding species at least some of which are fastened to or adapted to be fastened to the surface. In this embodiment, the surface has a chemical functionality that substantially inhibits non-specific binding of binding species that are not fastened to or adapted to be fastened to the surface, or aggregate-forming or fibril-forming species. This embodiment allows for particularly sensitive detection techniques. As used in this summary of the invention, "binding species" means binding species capable of binding a neurodegenerative disease aggregate-forming or fibril-forming species.

Another kit of the invention includes an article having a surface and a plurality of binding species fastened to or adapted to be fastened to the surface, and in this embodiment, the article is specifically not an SPR component. This embodiment allows for very sensitive, non-SPR analyses such as electronic analyses of diseased samples or drug activity.

In another aspect, the invention provides a series of components, systems, and articles that can be used in conjunction with various kits and methods of the invention. One article of the invention includes a surface at which a binding species is fastened. A signaling element also is fastened to the surface of this article. A composition of the invention includes a binding species, and an electronic signaling entity immobilized with respect to the binding species.

Another composition of the invention includes a binding species fastened to a moiety that can

coordinate a metal.

One system of the invention comprises at least two particles immobilized relative to a neurodegenerative disease fibril or aggregate. Another system of the invention includes at least two particles immobilized relative to a neurodegenerative disease aggregate-forming or fibril-forming species. These systems typically are the result of assays designed to detect diseased or pre-diseased states, or candidate drug screening mixtures.

In another aspect, the invention provides a series of methods. One method involves providing a binding species, fastened to or adapted to be fastened to a surface of an article, and converting the binding species from a state in which it is not a neurodegenerative disease aggregate-forming or fibril-forming species to one that is an aggregate-forming or fibril-forming species. This method finds significant use in highly sensitive assays, as will be apparent from the detailed description below.

In another embodiment, a method of the invention involves allowing a binding species to interact with a neurodegenerative disease aggregate-forming or fibril-forming species and thereby to be converted to a neurodegenerative disease aggregate-forming or fibril-forming species. The converted species thereafter participates in agglomeration characteristic of the presence of neurodegenerative disease aggregate forming or fibril-forming species. This agglomeration then is detected. This method also represents a significant advance in sensitivity of disease state or pre-disease state screening.

In another embodiment, a method of the invention is provided which, while not intended to be limited to this purpose, can find use in drug screening and represents a significant advance in that arena. The method involves providing a binding species fastened to or adapted to be fastened to a surface of an article, optionally allowing the binding species to fasten to the surface (if unfastened initially), and allowing the binding species to bind a neurodegenerative disease aggregate-forming or fibril-forming species. The binding can occur before or after fastening of the binding species to the surface, and the binding species is not converted to an aggregate-forming or fibril-forming species during binding. A second binding species, fastened to or adapted to be fastened to a surface of a second article, is allowed to bind the aggregate-forming or fibril-forming species. The binding of the second binding species to the aggregate-forming or fibril-forming species can occur at any point in time relevant to other steps according to this

method.

Another method of the invention involves an article having a surface and a plurality of binding species fastened to or adapted to be fastened to the surface. The surface of the article is exposed to a sample suspected of containing neurodegenerative disease aggregate-forming or fibril-forming species. A variety of individual techniques can accompany this method, for example, for detection of disease state in a sample, as will be apparent.

Another method of the invention involves first forming neurodegenerative disease aggregates or fibrils in a sample that contains aggregate-forming or fibril-forming species. The aggregates or fibrils are exposed to an article including a plurality of binding species fastened to or fastenable to the surface of the article. The binding species are capable of binding the aggregates, fibrils, aggregate-forming species, or fibril-forming species.

In another aspect, the invention provides a technique for determining effectiveness of candidate drugs for inhibition of neurodegenerative disease. The technique involves exposing a cell adapted to secrete neurodegenerative disease fibril- or aggregate-forming species to a candidate drug and determining the aggregation potential of material secreted from the cell. The cell can be adapted to secrete aggregate- or fibril-forming species by transcription with an overexpression plasmid of, for example, A $\beta$  fibril-forming species. The potential of the material secreted from the cell for aggregation characteristic of neurodegenerative disease can be monitored by exposing the cell environment, after exposing the cell to the candidate drug, to any assays of the invention, for example, assays designed to exhibit colloid agglomeration indicative of the presence of aggregate-forming or fibril-forming species.

Another aspect of the invention is a series of methods for forming a self-assembled monolayer on a surface. These methods can be used to form monolayer-coated articles that can be used in conjunction with other aspects of the invention. One method involves forming a self-assembled monolayer on a surface by exposing the surface to a medium containing self-assembled monolayer-forming molecular species and a surfactant. In another method, a surface is exposed to a medium containing self-assembled monolayer-forming molecular species and a carboxylate. In yet another method, surfactant and carboxylate can be included together in the medium. Surfactants that can be used in accordance with this aspect of the invention to deposit a self-assembled monolayer onto a species include essentially any of a wide variety of surfactants.

Carboxolates used to assist in self-assembled monolayer formation include preferably salts of carboxylic acids, including sodium citrate.

Another method for self-assembled monolayer formation involves forming a self-assembled monolayer on a surface of a colloid particle. The formation does not occur during  
5 formation of the colloid particle itself. For example, the monolayer can be formed on a fully pre-formed colloid particle. In another method, a self-assembled monolayer is formed on a surface of a colloid particle in suspension in a fluid, where the particle is not present at any fluid-fluid interface.

In more specific examples of various aspects of the invention, peptides or other binding  
10 species capable of incorporating into, or binding, aggregates plaques and fibrils are modified with signaling moieties and then mixed with a biological sample that may contain disease-associated peptide aggregates/fibrils or agents capable of inducing aggregate formation. Other species, capable of incorporating into plaques and fibrils, modified with recruitment moieties that can be differentially attracted to a sensing surface, are also mixed into the same sample.

15 After a suitable incubation period, the sample solution is subjected to conditions that differentially attract the recruitable peptides to a detection apparatus. The incorporation of signaling and recruitable moieties into pre-existing plaques or fibrils provides a sensitive and efficient method for labeling the aggregates and then concentrating them into a detection area.

The sequences of the binding species attached to signaling and recruitment species can  
20 be chosen such that they can readily incorporate into an existing fibril or aggregate, but in the absence of a pre-existing fibril, would not readily aggregate. Peptides can be linked to signaling moieties by direct chemical bond attachment to labeled dendrimers or polymers. Alternatively, peptides can be linked to signaling moieties through association with a particle-like material, including colloids, that simultaneously present both binding species and signaling group. In  
25 addition, versions of species that can participate in fibril formation can be added, free in solution (labeled or unlabeled), to accelerate the aggregation process and thus act as amplifiers.

Binding species can be linked to recruitment moieties by direct chemical attachment, such as attaching peptides to magnetic polymers that can be recruited to a detection area by an electromagnetic field or stationary magnet. Additionally, binding species can be linked to  
30 recruitment moieties by association with particle-like materials, such as magnetic beads, A-



sepharose beads, (which present a surface functionality including a portion of protein A that will bind antibodies), or charged particles, that can then be differentially attracted to a sensing area or surface. Binding species can also be linked by direct chemical bond or by association with particle-like materials to recruitment moieties that are recruitable by virtue of a biological recognition unit, such as a peptide linked to a metal binding tag (such as a histidine tag) which then binds to a metal coordinated by a surface-bound chelate, such as nitrilotriacetic acid (NTA) which can be immobilized at a surface as part of a self-assembled monolayer (SAM) at a surface. Binding species or any other species can also be linked to a surface with use of a Strept tag, commercially available from Biometra, attached to self-assembled monolayer-forming species such as a thiol. Strept tag forming part of a self-assembled monolayer will readily fasten biotin-modified moieties such as biotin-modified binding species. Binding species can be linked to surfaces in other ways, as described more fully below.

Other advantages, novel features, and objects of the invention will become apparent from the following detailed description of the invention when considered in conjunction with the accompanying drawings, which are schematic and which are not intended to be drawn to scale. In the figures, each identical or nearly identical component that is illustrated in various figures is represented by a single numeral. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention.

#### Brief Description of the Drawings

Figure 1 depicts peptides capable of participating in fibril or plaque formation attached to metal-containing compounds, such as ferrocene, via dendrimers or polymers. These derivatized peptides become associated with magnetic particles presenting similar peptides through their simultaneous incorporation into neuro peptide aggregates or fibrils. After recruitment to a SAM-coated electrode via a magnet, the presence of the aggregates is determined by detecting the presence of incorporated electroactive signaling moieties, like ferrocene, using techniques such as cyclic voltammetry (CV) or alternating current voltammetry (ACV).

Figure 2 depicts peptides capable of participating in fibril formation and a metal complex, such as a ferrocene derivative, attached to a colloid particle. These peptides become associated with magnetic particles bearing similar peptides when both are incorporated into

aggregates of abnormal versions of these prion-type (or fibril forming) peptides in the solution. After recruitment to a SAM-coated electrode via a magnet, the presence of the aggregates is electronically detected.

Figure 3 is a bar graph showing the detection of fibril formation by spectrophotometric means.

Figure 4 is a photocopy of a photograph of a negative control in one assay of the invention.

Figure 5 is a photocopy of aggregates, easily visibly identifiable to the unaided human eye, formed of an assay of the invention.

Figure 6 is a photocopy of a photograph of small aggregates visible under a microscope, formed in an assay of the invention from 50 picomolar A $\beta$  fibrils (figs. 4-6 all are magnified 40x).

Figure 7A is a photocopy of a photograph of a 40x magnified large colloid agglomeration, readily visibly identifiable to the unaided human eye, formed in accordance with another assay of the invention.

Figure 7B is a photocopy of a photograph of the negative control of the assay of Figure 7A.

Figure 8 is a photocopy of a photograph of an ELISA plate showing the results of a drug screening assay of the invention.

Figure 9 is an overlay of four ACVs showing electronic detection of a fibril in another assay of the invention.

Figure 10 shows the results of a light-scattering fibril assay of the invention.

Figure 11 is the negative control of the light-scattering experiment of Figure 10.

#### Detailed Description of the Invention

#### Definitions

"Small molecule", as used herein, means a molecule less than 5 kiloDalton, more typically less than 1 kiloDalton.

The term "neurodegenerative disease aggregates or fibrils" refers to complexes of the homologous molecules (*e.g.* fibril aggregates) as well as mixtures of molecules (*e.g.* interacting molecules such as binding partners), typically proteinaceous molecules, that are characteristic

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and expected to cause neurodegenerative disease. Aggregates of the present invention are typically of such a size (by virtue of the colloid interaction) that they can be visualized by eye, under a microscope, by light scattering, or by color changes.

The phrase "render the fibril visible" refers to techniques whereby a non-visible fibril is made visible to the eye, visible under a microscope, or visible by absorbance. A variety of approaches are contemplated, including but not limited to attaching or decorating fibrils with colored particles.

The term "candidate drug" as used herein, refers to any medicinal substance used in humans or other animals. Encompassed within this definition are compound analogs, naturally occurring, synthetic and recombinant pharmaceuticals, hormones, antimicrobials, neurotransmitters, etc. This includes any substance (whether naturally occurring, synthetic or recombinant) which is to be evaluated for use as a drug for treatment of neurodegenerative disease or prevention thereof. Evaluation typically takes place through activity in an assay, such as the screening assays of the present invention.

A variety of types of particles can be used in the invention. For example, "fluid suspendable particle" means a particle that can be made to stay in suspension in a fluid in which it is used for purposes of the invention (typically an aqueous solution) by itself, or can be maintained in solution by application of a magnetic field, an electromagnetic field, agitation such as stirring, shaking, vibrating, sonicating, centrifuging, vortexing, or the like. A "magnetically suspendable" particle is one that can be maintained in suspension in a fluid via application of a magnetic field. An electromagnetically-suspendable particle is one that can be maintained in suspension in a fluid by application of an electromagnetic field (e.g., a particle carrying a charge, or a particle modified to carry a charge). A "self-suspendable particle" is a particle that is of low enough size and/or mass that it will remain in suspension in a fluid in which it is used (typically an aqueous solution) for at least 1 hour. Other self-suspendable particles will remain in suspension, without assistance, for 5 hours, 1 day, 1 week, or even 1 month, in accordance with the invention.

As used herein, a "binding species capable of binding a neurodegenerative disease aggregate-forming or fibril-forming species" includes any species such as a protein, peptide, sequence of either, antibody, Congo red, Thioflavin-T, or any species capable of the binding so

described. In the case of antibodies, this binding is site-specific. In other cases it can be non-specific. In the case of proteins or peptides, the binding typically involves non-specific  $\beta$ -sheet/ $\beta$ -sheet interactions. Binding species also can include peptides, fragments, or whole proteins that are homologous to naturally-occurring neurodegenerative disease aggregate- or fibril-forming species.

“Proteins” and “peptides” are well-known terms in the art, and are not precisely defined in the art in terms of the number of amino acids that each includes. As used herein, these terms are given their ordinary meaning in the art. Generally, peptides are amino acid sequences of less than about 100 amino acids in length, but can include sequences of up to 300 amino acids.

Proteins generally are considered to be molecules of at least 100 amino acids.

As used herein, a “metal binding tag” refers to a group of molecules that can become fastened to a metal that is coordinated by a chelate. Suitable groups of such molecules include amino acid sequences including, but not limited to, histidines and cysteines (“polyamino acid tags”). Metal binding tags include histidine tags, defined below.

As used herein, “chelate coordinating a metal” or metal coordinated by a chelate, refers to a metal coordinated by a chelating agent that does not fill all available coordination sites on the metal, leaving some coordination sites available for binding via a metal binding tag.

“Signaling entity” means an entity that is capable of indicating its existence in a particular sample or at a particular location. Signaling entities of the invention can be those that are identifiable by the unaided human eye, those that may be invisible in isolation but may be detectable by the unaided human eye if in sufficient quantity (e.g., colloid particles), entities that absorb or emit electromagnetic radiation at a level or within a wavelength range such that they can be readily detected visibly (unaided or with a microscope or the like), or spectroscopically, entities that can be detected electronically, such as redox-active molecules exhibiting a characteristic oxidation/reduction pattern upon exposure to appropriate activation energy (“electronic signaling entities”), or the like. Examples include dyes, pigments, electroactive molecules such as redox-active molecules, fluorescent moieties, up-regulating phosphors, or enzyme-linked signaling moieties including horse radish peroxidase and alkaline phosphatase.

As used herein, “fastened to or adapted to be fastened”, in the context of a species relative to a surface of an article, means that the species is chemically or biochemically linked

via covalent attachment, attachment via specific biological binding (e.g., biotin/streptavidin), coordinative bonding such as chelate/metal binding, or the like. For example, "fastened" in this context includes multiple chemical linkages, multiple chemical/biological linkages, etc., including, but not limited to, a binding species such as a peptide grown on a polystyrene bead, a binding species specifically biologically coupled to an antibody which is non-specifically biologically bound to a protein such as protein A, which is covalently attached to a bead, a binding species that forms a part (via genetic engineering) of a molecule such as GST or Phage, which in turn is specifically biologically bound to a binding partner covalently fastened to a surface (e.g., glutathione in the case of GST), etc. As another example, a moiety covalently linked to a thiol is adapted to be fastened to a gold surface since thiols bind gold very strongly, perhaps covalently. Similarly, a species carrying a metal binding tag is adapted to be fastened to a surface that carries a molecule covalently attached to the surface (such as thiol/gold binding) which molecule also presents a chelate coordinating a metal. A species also is adapted to be fastened to a surface if a surface carries a particular nucleotide sequence, and the species includes a complementary nucleotide sequence. The definition of "fastened" includes some non-specific binding, namely, intentional adherence of a species to a surface for purposes of a technique of the invention. Non-specific binding falling under the definition of "fastened" in the invention will result in attachment that withstands routine washing steps associated with assays of the invention. Specifically, a non-specifically bound species is "fastened" to a surface if it will withstand at least one routine washing with an aqueous wash solution typically including a surfactant such as Tween-20 and a buffer, such as a phosphate buffer for maintaining the fluid at physiological pH.

"Specifically fastened" or "adapted to be specifically fastened" means a species is chemically or biochemically linked to a surface as described above with respect to the definition of "fastened to or adapted to be fastened", but excluding all non-specific binding.

"Non-specific binding", as used herein, is given its ordinary meaning in the field of biochemistry.

"Colloids", as used herein, is given its ordinary meaning in the field of biochemistry. Typically, colloid particles are of less than 250 nm cross section in any dimension, more typically less than 100 nm cross section in any dimension.

A “moiety that can coordinate a metal”, as used herein, means any molecule that can occupy at least two coordination sites on a metal atom, such as a metal binding tag or a chelate.

As used herein, a component that is “immobilized relative to” another component either is fastened to the other component or is indirectly fastened to the other component, e.g., by being fastened to a third component to which the other component also is fastened, or otherwise is translationally associated with the other component. For example, a signaling entity is immobilized with respect to a binding species if the signaling entity is fastened to the binding species, is fastened to a colloid particle to which the binding species is fastened, is fastened to a dendrimer or polymer to which the binding species is fastened, etc.

“Neurodegenerative disease aggregate-forming or fibril-forming species”, as used herein, means biological species associated with neurodegenerative disease having sufficient binding capacity to bind to other molecules associated with neurodegenerative disease (including like molecules), to form fibrils or aggregates characteristic of neurodegenerative disease. Such aggregate-forming or fibril-forming species typically are characterized by a change in molecule conformation, relative to sequence-homologous, healthy counterparts, allowing them to bind more readily to like or similar molecules. In some cases, such aggregate-forming or fibril-forming species have the capability to convert binding species from non-aggregate-forming or fibril-forming conformation into aggregate-forming or fibril-forming conformation. Protofibrils have been reported to bind other molecules associated with neurodegenerative disease to form fibrils for aggregates characteristic of neurodegenerative disease. To the extent that this is the case, protofibrils are included in the definition of neurodegenerative disease aggregate-forming or fibril-forming species as used herein. The aggregate- or fibril-forming species typically include amino acid sequences from 1-38 to 1-44.

“Diverse biological species” means different animals, such as mouse and hamster, mouse and goat, etc.

The term “sample” refers to any cell, tissue, or fluid from a biological source (a “biological sample”, or any other medium that can advantageously be evaluated in accordance with the invention including, but not limited to, a biological sample drawn from a human patient, a sample drawn from an animal, a sample drawn from food designed for human consumption, a sample including food designed for animal consumption such as livestock feed, an organ

donation sample, or the like.

A "sample suspected of containing" a particular component means a sample with respect to which the content of the component is unknown. For example, a fluid sample from a human suspected of having neurodegenerative disease, but not known to have neurodegenerative disease, defines a sample suspected of containing neurodegenerative disease aggregate-forming or fibril-forming species. "Sample" in this context includes naturally-occurring samples, such as physiological samples from humans or other animals, samples from food, livestock feed, etc., as well as "structurally predetermined samples", which are defined herein to mean samples, the chemical or biological sequence or structure of which is a predetermined structure used in an assay designed to test whether the structure is associated with neurodegenerative disease. For example, a "structurally predetermined sample" includes a peptide sequence, random peptide sequence in a phage display library, and the like. Typical samples taken from animals include blood, urine, ocular fluid, saliva, cerebro-spinal fluid, fluid or other samples from tonsils, lymph nodes, needle biopsies, etc.

As used herein, the term "poly-histidine tract" or "histidine tag" or "HIS-tag" refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a peptide or protein. A poly-histidine tract of six to ten residues is preferred for use in the invention. The poly-histidine tract is also defined functionally as being a number of consecutive histidine residues added to a protein of interest which allows the affinity purification of the resulting protein on a metal chelate column, or the identification of a protein terminus through the interaction with another molecule (e.g. an antibody reactive with the HIS-tag). Histidine-tagged ligands and His-tagged putative binding partners can be incubated together with NTA/Ni(II) presenting colloids. A visible reticulum will result if the two components are binding partners. Alternatively, the putative binding partners can be GST fusion proteins that would bind to glutathione presented on the colloid. Other linkers useful for attaching a binding species or other participant in assays of the invention to a surface include affinity tags. Affinity tags are well-known species used widely in biology, biochemistry, etc.

Referring now to Figure 1, one arrangement of the present invention is illustrated schematically. Shown in Figure 1 is an article 20, specifically an electrode including a SAM 22 on a surface thereof. SAM 22 includes "molecular wires" 24, in embodiments where it is

desirable that SAM 22 be relatively electrically conductive. Typically, a mixed SAM is formed of a relatively non-conducting species such as an alkyl optionally terminating in a non-specific binding inhibitor such as polyethylene glycol, mixed with molecular wires. The selection of surfaces and functional groups for binding self-assembling monolayer molecules to surfaces is a well-known art. See U.S. Patent Nos. 5,512,131 and 5,620,850, and International Patent Publication no. WO 96/29629, incorporated herein by reference, for this and other teachings.

"Molecular wires" as used herein, means wires that enhance the ability for a fluid encountering a SAM-coated electrode to communicate electrically with the electrode. This includes conductive molecules or, as mentioned above and exemplified more fully below,

molecules that can cause defects in the SAM allowing fluid contact with the electrode. A non-limiting list of additional molecular wires includes 2-mercaptopyridine, 2-mercaptobenzothiazole, dithiothreitol, 1, 2-benzenedithiol, 1, 2-benzenedimethanethiol, benzene-ethanethiol, and 2-mercaptoethylether. Conductivity of a monolayer can also be enhanced by the addition of molecules that promote conductivity in the plane of the electrode.

Conducting SAMs can be composed of, but are not limited to: 1) poly (ethynylphenyl) chains terminated with a sulfur; 2) an alkyl thiol terminated with a benzene ring; 3) an alkyl thiol terminated with a DNA base; 4) any sulfur terminated species that packs poorly into a monolayer; 5) all of the above plus or minus alkyl thiol spacer molecules terminated with either ethylene glycol units or methyl groups to inhibit non specific adsorption. Thiols are described because of their affinity for gold in ready formation of a SAM. Other molecules can be substituted for thiols as known in the art from U.S. Patent No. 5,620,820, and other references. Molecular wires typically, because of their bulk or other conformation, creates defects in an otherwise relatively tightly-packed SAM to prevent the SAM from tightly sealing the surface against fluids to which it is exposed. The molecular wire causes disruption of the tightly-packed self-assembled structure, thereby defining defects that allow fluid to which the surface is exposed to communicate electrically with the surface. In this context, the fluid communicates electrically with the surface by contacting the surface or coming in close enough proximity to the surface that electronic communication via tunneling or the like, can occur.

A particle 26, specifically a magnetic bead, is provided along with a plurality of binding species, capable of binding a neurodegenerative disease aggregate-forming or fibril-forming



species, fastened to or adapted to be fastened to the bead. A plurality of binding species 28 also are provided fastened or fastenable to signaling entities 30 which, in the embodiment illustrated, comprise dendrimers 32, each carrying a plurality of individual signaling entities 34. A plurality of target molecules 36, which are neurodegenerative disease aggregate-forming or fibril-forming species, are introduced by, for example, being included in a physiological sample suspected of containing the molecules. Binding species 28 bind to aggregate-forming or fibril-forming species 36 to define a linkage including bead 26 with attached binding species, signaling entity 30 with attached binding species, and aggregate or fibril-forming species 36 immobilized with respect to each. This arrangement can be drawn to a surface of electrode 20 via a magnet 38 under the electrode and, where signaling entities 34 are electronic signaling entities, their proximity near electrode 20 can be detected indicating presence of the aggregate or fibril-forming species 36 in the sample.

Specifically, in this embodiment, electronic signaling entity 34 can be a redox-active molecule such as a metallocene, specifically ferrocene. Proximity of a ferrocene as signaling entity 34 near electrode 20 can be determined by a cyclic voltametric technique such as alternating current voltammetry (ACV).

Binding species 28 need not be fastened to signaling entity 30 and/or bead 26 initially, but a mixture can be provided including a sample suspected of containing species 36, binding species 28, signaling entity 30, and bead 26, where binding species 28 is adapted to be fastened to signaling entity 30 and/or bead 26 if not already fastened. In this arrangement, species 28 can be adapted to be fastened to signaling entity 30 and/or bead 26 by including a chemical or biological binding partner of a molecule fastened to signaling entity 30 or bead 26. Bead 26, if a polymeric bead, can include a variety of covalently-attached linking molecules. Similarly, entity 30 can be modified in this way. Bead 26 also can be coated with a surface layer of a material that facilitates formation of a SAM thereon, such as gold, and a SAM forming molecule (such as a thiol in the case of the gold surface) carrying a linking molecule can be formed thereon. Linkages between a linking molecule on bead 26 or signaling entity 30 and binding species 28 can include metal binding tag/metal/chelate linkages, complementary nucleic acid sequences, biotin/streptavidin, etc. In an arrangement where a metal binding tag/metal/chelate linkage is used, a chelate can form part of a SAM on bead 26, and can be covalently attached to signaling

entity 30. The chelate coordinates a metal, but leaves at least two open coordinate sites on the metal. A metal binding tag such as a polyamino acid tag can be incorporated into binding species 36, giving species 36 the ability to fasten to the bead or signaling entity by coordination of the metal binding tag to the metal. Examples of suitable chelates include nitrilotriacetic acid, 2,2'-bis(salicylideneamino)-6,6'-demethyldiphenyl, or 1,8-bis(a-pyridyl)-3,6-dithiaoctane. In an alternate immobilization technique, binding species 36 can carry a terminal cysteine and fasten thereby to a gold surface of bead 26.

A variety of binding species 36 can be selected. The binding species can be a peptide, protein, sequence from a protein, small molecule such as Congo red or Thioflavin-T, or sequence homologous to sequence derived from aggregate-forming species, RNA, DNA, nucleoside derivatives, or antibody to the aggregate-forming or fibril-forming species 36.

A variety of surfaces can form components of kits or systems or participate in methods of the invention. Surfaces such as surfaces of beads, colloids, electrodes, ELISA plates, other multi-well plates, and the like can be used.

In many techniques of the invention it is desirable to prevent any binding species introduced into an assay from participating in aggregate formation. In such a case, aggregate-binding, but aggregate-formation-resistant binding species can be used. Aggregate-formation-resistant species can easily be selected by those of ordinary skill in the art from among species such as antibodies, fragments of proteins or peptides, etc. that will bind but not form aggregates.

The surface of the article, whether it be an electrode, particle, bead, or the like, preferably has a chemical functionality that substantially inhibits non-specific binding of aggregate-forming or fibril-forming species. This can be accomplished in one embodiment by forming a SAM on the surface for presentation of a desired functionality for the assay, the SAM being a mixed SAM including other non-specific-binding inhibitors. NSB inhibitors include polyethylene glycol-terminated SAM-forming species.

Preferably, a SAM coating the colloid inhibits colloid/colloid self-aggregation. This can be accomplished by using a sufficient amount of SAM-forming species preventing charges moieties and/or carboxy-terminated species outward in the SAM. The charged moieties and/or carboxy-terminated species should be present in an amount of about 10 percent or more of the SAM, or preferably at least about 30 percent or at least about 45 percent of the SAM.

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Referring now to Figure 2, another arrangement suitable for use as an assay as illustrated in Figure 1 is shown, but with modifications. In the arrangement of Figure 2, rather than a signaling entity 30 fastened to or adapted to be fastened to species 28, species 28 is immobilized relative to or adapted to be immobilized relative to individual signaling entities 34 by way of each of species 28 and signaling entities 34 being fastened to or adapted to be fastened to a colloid particle 40. Species 28 and signaling entities 34 can be adapted to be fastened to colloid particle 40 by any of a variety of chemical or biological techniques known in the art or described herein. It is particularly convenient, with the use of gold colloid particles 40, to attach signaling entities 34 and binding species 28 to a thiol which will bind to the surface of colloid 40. A SAM can be formed coating a surface of colloid 40 including the signaling entities 34 and binding species 28. In the arrangement of Figure 2, binding of binding species 28, fastened to each of bead 26 and colloid 40, to aggregate or fibril-forming species 36, followed by activation of magnet 38 to draw bead 26 to the proximity of electrode 20, in turn draws signaling entities 34 close to electrode 20 since signaling entities 34 are immobilized relative to bead 26. Electronic detection of signaling entities 34 near electrode 20 is routine using ACV or the like.

The assays illustrated in Figures 1 and 2 can be used for a variety of purposes. When used to determine whether aggregate or fibril-forming species 36 is present in a sample, a kit including bead 26, signaling entity 30 (Figure 1) or colloid particle 40 (Figure 2), and binding species 28 fastened to or adapted to be fastened to bead 26, entity 30, or colloid particle 40, is provided and mixed in solution with the sample. If aggregate or fibril-forming species 36 is present in the sample, then this is indicated by the proximity of signaling entity 34 near electrode 20 after mixture and activation of magnet 38. If aggregate of fibril-forming species 36 is not present, then signaling entity 34 should not be drawn to the electrode upon activation of the magnet.

In another technique, used for drug screening, aggregate or fibril-forming species 36 can be provided in known quantity in the sample in addition to a candidate drug for treatment of neurodegenerative disease. In such an arrangement, different candidate drugs will have different effects upon the binding or signaling entity 30 or colloid particle 40 to bead 26 via the aggregate or fibril-forming species 36. Candidate drugs that inhibit this binding are candidates for treatment of neurodegenerative disease. The assay can be established in such a way that a signal

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(proximity of signaling entity 34 to electrode 20) is proportional or otherwise related not only to the concentration of aggregate or fibril-forming species 36 in the sample, but to the degree of aggregation or fibril formation occurring within the sample. In such an arrangement, in a drug screening assay, the signal will be proportional or related to the degree to which a particular drug candidate prevents aggregation or fibril formation characteristic of neurodegenerative disease from aggregate or fibril-forming species 36 present in the sample.

In drug screening assays, it is often desirable to quantitatively determine the relationship between immobilization of bead 26 relative to signaling entities 34 in conjunction with concentration of aggregate or fibril-forming species 36 and/or concentration of drug candidate in a particular solution. In such an arrangement, it is desirable that binding species 28 bind aggregate or fibril-forming species 36 without conversion of binding species 28 to an aggregate-forming or fibril-forming species, to maintain a 1:1 correlation between binding events and signal. This can be done by selecting binding species 28 and aggregate or fibril-forming species 36 from diverse biological species, e.g. from hamster and mouse, respectively, or a sequence derived from the same species that binds 1:1 with target.

In another arrangement, it is desirable to maximize sensitivity of the assay, rather than maintaining a 1:1 correlation between the binding events and signals or species in solution. In such an arrangement it is desirable to convert binding species 28 from species that simply will bind neurodegenerative aggregate or fibril-forming species 36, but are not aggregate or fibril-forming species themselves, into aggregate or fibril-forming species with the ability to convert other binding species to aggregate or fibril-forming species. In such an arrangement, with references to Figures 1 and 2, if only a very, very small amount of aggregate or fibril-forming species 36 is present in a sample (e.g. where as few as 100,000 molecules must be detected because that number of infectious prion molecules is sufficient to cause disease, characteristic of a sample from a patient in a very early stage neurodegenerative disease), "amplifier" species 29 can be provided in solution and mixed with a sample. In such an arrangement, even though a very small amount of aggregate or fibril-forming species 36 may be present in a sample, amplifier species 29 can be converted thereby to aggregate or fibril-forming species thereby rendering aggregate or fibril-forming species present in sufficient concentration to be detected using the assay. If no aggregate or fibril-forming species 36 is initially present in the sample,

then amplifier species 29 will remain unconverted and little or no signal will be detected. Not only can amplifier species 29 amplify the concentration of aggregate or fibril-forming species 36 present in a sample to detectable levels, but it can facilitate massive aggregation of species 36 characteristic of neurodegenerative disease which in turn can form macro structures including many hundreds or thousands of beads, colloids, and/or signaling entities bound to many thousands of aggregate or fibril-forming species in a single macro structure. These structures can be easily detected electronically, and often visibly.

Amplifier species 29 can be selected among all of the species listed above for binding species 28, but need not be the same as binding species 28 in a particular assay. For example, binding species 28 in a particular assay can be a binding species, but without the ability to be converted to an aggregate or fibril-forming species, while amplifier species 29 can be convertible to define an aggregate or fibril-forming species. The latter situation (binding species 28 is not convertible but amplifier species 29 is convertible to a fibril or aggregate-forming species) it is useful in preventing particle/particle aggregation in the absence of a positive result (in the absence of aggregate or fibril-forming species 36 in a sample), but amplification of species 36, when it is provided in a sample, occurs, sensitizing the assay.

In conjunction with the use of amplifier species 29 to cause massive aggregation of a sample that initially includes insufficient aggregate or fibril-forming species 36 to cause massive aggregation (yet levels of species 36 desirably detected), electronic signaling entities and magnetic beads as illustrated in Figures 1 and 2 need not necessarily be used. In such an arrangement, a kit can be provided including colloid particles 40, binding species 28 fastened to or adapted to be fastened to colloid particles 40, and amplifier binding species 28. Such a kit can be mixed with a sample suspected of containing aggregate or fibril-forming species 36. Even at very low levels, species 36 can convert amplifier binding species 28 to aggregate or fibril-forming species, causing massive agglomeration and the formation of macro structures easily detectable, for example, visible by the unaided human eye, or visible under a microscope, detectable via a light scattering, absorbance or the like. In this arrangement, colloid particles 40 need not necessarily carry any auxiliary signaling entities.

In the arrangement described above with reference to Figures 1 and 2, it is desirable that binding species 28 be selected, and provided in concentration on surfaces of beads 26 and

colloid particles 40 (where colloid particles are used), such that in the absence of auxiliary, non-surface immobilized aggregate-forming or fibril-forming species 36, particle agglomeration on particle/particle exposure is hindered within a time frame allowing comparison of (1) agglomeration in the absence of auxiliary aggregate or fibril-forming species with (2) agglomeration in the presence of auxiliary aggregate or fibril-forming species. That is, if no aggregate or fibril-forming species 36 is initially present in a sample, then agglomeration of colloid particles may occur, but only slowly or to a limited extent, whereas if species 36 initially is present, then agglomeration will occur more rapidly and/or to a much greater extent. In such an assay a control can include no sample, and the rate and/or agglomeration level of the sample can be compared to that of the control.

It is not intended that the methods and assays of the present invention be limited to only a particular fibril associated with a particular neurodegenerative disease. Indeed, the present invention contemplates detecting fibrils associated with a host of such diseases (*e.g.* Alzheimer's, Parkinson's, Creutzfeldt Jakob, etc.).

The present invention provides a variety of assays and components for assays, for detection of disease or determination of effectiveness of drugs for treatment of such disease. The assays can involve electronic interaction of a sample or drug screen assay with a sensing electrode, or can involve aggregation, which can be detected by a variety of techniques, including but not limited to visual inspection, density scanning, light transmission, absorbance, color change and/or light scattering. In connection with aggregation, binding species that can bind aggregate-forming or fibril-forming species or can incorporate into pre-existing aggregates or fibrils, are attached to colloids (*e.g.* gold colloids) or other particles (*e.g.* fluorescent beads). These particles, optionally along with free binding species (species that are not attached to colloids but are suspended or dissolved in a fluid in which the colloids are suspended) are mixed with solutions that contain aggregates or fibrils (*e.g.* patient samples). As the particles (*e.g.* colloids) become incorporated into the fibril, they render the fibril optically detectable. To inhibit peptides immobilized on colloids from interacting with each other, repellant groups - such as charged moieties (COOH, PNA, charged peptides) - can be fastened to surfaces of the colloids. This causes the immobilized peptides to interact with the fibril rather than with each other and thus reduces the occurrence of false positives in a diagnostic assay.

Also, flexible groups, such as glycol units can be chemically inserted between the nitrilo tri-acetic (NTA) moiety and the thiol portion. The introduction of more degrees of freedom would slow the rate of interaction by increasing the entropic cost of the reaction.

It is not intended that the present invention be limited by the method of detection. To facilitate

5 detection, the present invention contemplates a variety of techniques. For example, a signaling entity, such as a dye, can be fastened to one end of a peptide capable of incorporating fibrils and plaques. As the signal-modified peptides incorporate into the fibril, they render it "visible" by concentrating the dye on its surface. In another embodiment, fluorescent moieties and

quenching moieties are attached separately to the ends of peptides. At low concentration in

10 solution, they do not interact frequently with each other; however, when co-localized on a fibril, a quenching reaction takes place, causing a change in the fluorescence of the solution. These changes can be analyzed using standard ELISA plate readers. In yet another embodiment, two separate compounds are attached to the N-terminus end of a peptide capable of incorporating into a fibril or plaque. The compounds are designed such that one compound modifies the

15 second to render it active or detectable. The chemical modification is such that without the modification the compound does not fluoresce but after modification it does. Statistically, the reaction will happen orders of magnitude more frequently when the two compounds are co-localized on a fibril or plaque. In another embodiment electronic interaction serves as a

detection technique, for example, where the presence of a redox-active molecule near an

20 electrode surface can be detected, indicating binding of a species immobilized relative to the redox-active molecule to a species immobilized relative to the electrode, or relative to a recruitable particle that can be drawn to the electrode, such as a magnetic bead. In another

detection technique, an image of a sample can be digitized, then pattern recognition software is used to determine whether the sample contains aggregates. For example, a sample as shown in

25 Figure 8 can be digitized using a CCD camera, and pattern recognition can be used to determine color, aggregate size, aggregate spatial distribution, relative amount of aggregation, etc. This allows automation of both diagnostic screening and drug screening. It is particularly useful, when doing screening of a large number of drugs, to be able to individually spatially address various samples. Techniques of the invention can be carried out using individually spatially-

30 addressable regions, such as different wells of a multi-well plate (see Figure 8) and/or a plurality

of electrodes. A plurality of electrodes can be arranged in individual wells of a multi-well plate, or the like.

It is particularly useful, when doing screening of a large number of drugs, to be able to individually spatially address various samples. Techniques of the invention can be carried out using individually spatially-addressable regions, such as different wells of a multi-well plate (see Figure 8) and/or a plurality of electrodes. A plurality of electrodes can be arranged in individual wells of a multi-well plate, or the like.

Regardless of which embodiment is employed, the assays of the present invention can be readily adapted to screen drug libraries for compounds that inhibit aggregate or fibril formation.

For a drug-screening assay, one can attach binding species to colloids (or other particle) and incubate with solutions containing aggregate-forming or fibril-forming species and a drug candidate. The solutions may or may not be agitated to accelerate the incorporation of peptide into the fibrils. As the binding species are incorporated into the aggregates or fibrils, or aggregate-forming or fibril-forming species that then form aggregates or fibrils, they bring the attached colloids close to each other, which causes the colloid solution to change color (e.g. from a pink suspension to a dark blue precipitate in a clear solution). This transition is clearly visible by eye. By absorption spectrophotometry, the peak at 569 nm degrades as the colloids aggregate.

In one embodiment, solutions that contain 1  $\mu$ M  $\beta$ -amyloid (A $\beta$ ) fibril 46 "seeds" and His-tagged  $\beta$ -amyloid peptides attached to gold colloids, and control solutions that did not contain the fibril seeds, are subjected to absorption spectrometry. Both show the major absorption peak at 569 nm. After the initial scan, the reaction containing means (e.g. a cuvette, tube, microwell, etc.) is agitated then rescanned. When peptides are attached to colloids at much lower density, the colloids do not precipitate out of solution or change color, but act to decorate the fibril with visible particles. Dark red clusters appear in a cleared solution and are clearly visible by eye. Enhanced resolution can be obtained by observing the solutions under 60-100X magnification. The analysis process can be automated by using (by way of example only) absorption spectrometry or light scattering, which can distinguish large fibrillar structures from small particulate matter.

In one embodiment, the method of the present invention employs peptides (that can form



fibrils characteristic of neurodegenerative diseases) which are modified with signaling and/or recruitment moieties and are incubated with a biologically-derived sample (or an in vitro mimic of such a sample) potentially containing converted or aggregated peptides indicative of the disease state. The existence of peptide plaques, aggregates, or fibrils is detected via the incorporation of the labeled peptides. This assay can be done in a variety of ways, as described below, in a system that allows fast and efficient detection of fibrillar or aggregated species.

For example, one element can be a magnetic particle that bears a peptide that participates in fibril or plaque formation. The second element can be in the form of a colloid derivatized with a heterologous SAM that presents both a peptide that participates in fibril or plaque formation and a metal-containing compound, such as ferrocene, which acts as a signaling moiety. Peptides that participate in fibril or plaque formation can catalyze the misfolding and spontaneous aggregation of other peptides, such as those attached to the beads and colloids. The presence of these fibrillar or converted species of peptide can be detected in a sample by detecting the association of peptides on magnetic beads with peptides on signaling colloids through their simultaneous incorporation into fibrils or aggregates.

Following an incubation period in which this aggregation is allowed to occur, an electromagnetic field can be applied that attracts the complex, which now contains magnetic particles as well as signaling colloids. In this system, magnetic particles provide a method of mixing biological recognition particles through a large sample volume and then rapidly recruiting the labeled targets to an electrode for detection. When an oscillating potential is applied to the electrode, a current peak at the oxidation potential that is characteristic of the included metal indicates the presence of the aggregate at the electrode and, consequently, the presence of the aggregated peptides. In addition, free non-converted peptides can be added into the measurement solution to increase the rate of aggregation and/or the amount of material aggregated, thus acting as aggregation amplifiers. Aggregate formation can also be accelerated by varying the incubation and measurement conditions, including using frequency pulses and changes in temperature and electrical and magnetic fields.

The complexes can be detected using a variety of techniques, however, the preferred detection method is alternating current voltammetry (ACV). This detection method can be supplemented by the use of additional analysis techniques such as higher order harmonic

analysis. These complexes may also be able to be detected by optical means such as surface plasmon resonance (SPR). In using the latter technique, large shifts in optical properties are caused by recruitment of the complexes to the detecting surface.

The aggregated state of these disease related peptides can also be detected by attaching  
5 potentially pathogenic peptides (binding species or aggregate or fibril-forming species) to either end of a linker, such that upon introduction of a polymerization seed or aggregate (aggregate or fibril-forming species optionally able to convert binding species to aggregate or fibril-forming species), the chains would form aggregates whose detectable properties would be altered from the original, non-aggregated state, e.g. by being visibly identifiable, rendered able to scatter light  
10 in light-scattering detection, change in viscosity in a flow detection system, electronic detection where an electronic signaling entity is linked to a component that forms part of the aggregate, etc. Alternatively, normal forms of pathogenic peptides can be attached to functionalized hydrogels or to ordered "holes" in gel-like materials. Since the introduction of a seed or protein aggregate would cause the conversion of the presented, soluble peptides into polymers or  
15 aggregates, that have altered optical properties, these aggregates could be optically detected.

Alternatively, the magnetic particle can be gold-coated and derivatized with a SAM that presents the desired peptide either by direct attachment of a peptide-thiol, or indirectly such as a DNA-peptide binding to a DNA-thiol incorporated in a SAM, or a histidine-tagged peptide binding to an NTA-thiol incorporated into a SAM. The colloid described herein may be a  
20 cluster of metal atoms, the preferred metal being gold. Because gold clusters can be made from as few as 20 gold atoms, colloids would be among the smallest possible particles with a diameter of only a few nanometers which could have significant advantages when incorporating these relatively small-sized fibril-forming peptides and metal-containing compounds into fibrils and plaques and maintaining them in suspension.

25 The magnetic particle can also be gold-coated and derivatized with a SAM that includes NTA-thiols for the capture of histidine-tagged peptides and peptide fragments. The SAMs can also be derivatized with DNA-thiols that are bound to a complementary DNA sequence that is also covalently attached to a peptide. There can also be a linker inserted between the peptide and the particle or colloid or the recognition group that links the peptide to the particle or colloid  
30 so that steric hindrance does not interfere with the interaction between the normal and abnormal

prion peptides. This linker or spacer can be made of a variety of materials, including glycols or amino acids. The length of the spacer can be varied to promote a shift in the oxidation potential of the electroactive signal detection substance by creating an increased hydrophobic environment due to the proximity of the aggregates.

5           The magnetic particle can also be replaced by a second colloid of larger dimensions and these larger colloids can be electromagnetically attracted to the electrode surface. However, they will only signal if decorated with satellite colloids that present signaling moieties. Alternatively, either colloid can be decorated with charged moieties that alter its mobility when an electromagnetic force is applied. All of the colloids can also be of similar size and an  
10       electromagnetic force can then be used to separate aggregated colloids from individual particles. In another variation, the first particle could be a heavier particle or colloid that can be mechanically mixed during incubation and subsequently allowed to accumulate on the electrode surface by sedimentation when measurements are taken.

          These techniques can also be employed in a one-particle system. For example, magnetic  
15       beads can present pathogenic peptides and a transition metal complex. When pathogenic peptides in solution interact with peptides on beads to form aggregates, the local environment of the transition metal changes such that it will oxidize at a higher potential. The presence of pathogenic aggregates or fibrils are detected as a shift in the oxidation potential of the transition metal or as the appearance of a second oxidation peak at a lower potential, when the system is  
20       electrochemically analyzed. An alternative one-particle system includes magnetic particles that display the normal peptide and amplifying peptides in solution that carry transition metal complexes. These transition metals can be linked to dendrimers or polymers, either conducting or non-conducting. As pathogenic peptides in the test sample promote aggregate formation, the metal-containing compounds become intertwined with peptides attached to magnetic particles  
25       resulting in an electroactive and magnetic complex that can be recruited and electronically detected.

          This assay system can also be constructed without using beads. Peptides with metal-containing compounds or peptide-thiols and transition metal complex thiols can be immobilized on or incorporated into SAMs on electrodes. Amplifying peptides may or may not be added to  
30       the measurement solution. If the test sample contains pathogenic or infectious peptides, then

they attach to the surface immobilized peptides. This changes the local environment of the metal complexes and thus changes their oxidation potential. Amplifying peptides in the measurement solution may carry metal complexes that may be linked to dendrimers or polymers. If the test sample contains pathogenic peptides, then the aggregated species act to bridge the metalated peptides and the surface immobilized peptides. This interaction places the signaling moiety near the electrode surface and an electronic signal is transmitted to the electrode. Similarly, peptides incorporated into SAMs, formed on electrodes by either direct thiol attachment or by binding a His-tagged peptide to a pre-formed NTA-SAM, can be introduced to a sample solution that contains pathogenic peptides and also colloids that present both peptide and signaling moieties such as ferrocenes. Again, their integration into fibrils/aggregates brings the signaling moiety (in this case a colloid) close to the electrode and a signal is transmitted. Conversely, peptides derivatized with NTA groups can be attached to surfaces via an interaction with a peptide that contains a six histidine stretch. Amplifying peptides may or may not be added to the measurement solution.

These techniques can also be adapted to screening for potential drug candidates to treat these neurodegenerative diseases. Specifically, in all of the described variations, a drug candidate can be added to the solution and its ability to inhibit aggregate formation as a function of time and concentration, which would translate into dosage, measured. Various parameters, such as a loss of signal or a shift in the position of the current peak can be indicative of a positive, and therefore therapeutically useful, effect. In addition, the drug candidates can be attached to beads bearing metal-containing compounds, which can be colloids coated with SAMs either with or without NTA-thiols, that oxidize at a different potential than those metal-containing compounds attached to the colloids having peptides attached to them. One then looks for the different oxidation peak. The signaling metal-containing compound can also be removed from the colloid that has peptides attached and the single current peak from the drug-containing colloids measured. Also, a multiplexing drug screening device can be designed in which the drug candidates are added to microarrays and the whole array is flipped upside down onto a SAM-modified electrode lid for analysis.

This competitive inhibition assay could also be performed in a system in which the signal detection compound is on a colloid containing drug candidates. In this system, a magnetic bead

without metal-containing signal detection compounds carries peptides. Free peptides in solution can interact with the peptides bound to these magnetic beads. Putative drug candidates are then placed on colloids having metal-containing signal detection compounds. The metal-containing signal detection compounds on the colloids would signal if they interrupted the aggregation process and interacted directly with the peptide on the magnetic beads. One could also obtain a signal if the drug candidates interacted with aggregates. The latter information could be useful, but would likely require a subsequent multi-step assay to determine where the drug was acting.

Drug candidates are added to solutions containing pre-formed fibrils, peptides attached to electronic signaling colloids, and peptides attached to magnetic beads. Following an incubation period, magnetic beads are attracted to a sensing electrode and analyzed by ACV. A loss of signal indicates that the drug candidate has inhibited fibril formation.

The meta-stable protofibril can be selectively formed and purified to homogeneity by size exclusion chromatography, then used as the target fibril species for drug screening. Similarly, alpha-synuclein, the protein that incorporates into fibrils characteristic of Parkinson's disease, can be His-tagged and attached to electronic signaling colloids and magnetic particles. These particles are incubated with synuclein fibrils in solution or from a Parkinson's patient sample, then magnetically attracted to a sensing electrode for electronic analysis.

In these assays, it can be helpful if peptides or proteins that are attached to surfaces (or the surfaces of particle-like entities) are presented away from the surface (separated from the surface) so that the interactions of interest to occur. The particle or surface can sterically interfere with binding processes necessary for the assay. A flexible linker can be inserted between the protein or peptide and the His-tag. Alternatively, the length of the carbon chain, to which the NTA group is attached can be varied, and a number of linker groups can be inserted between the NTA group and the carbon chain.  $HS-(R)_n-(X)_m-NTA$ , where S is sulfur, R is any molecule that can incorporate into a SAM such as  $CH_2$ , X is a linker unit such as ethylene glycol, and NTA is nitrilo tri-acetic acid. Pulsing the solutions with electromagnetic fields or mechanical agitation is used to accelerate the incorporation process.

As noted previously, it is also not intended that the assays of the present invention be limited to electronic interaction. In a particular embodiment, the method does not employ a sensing surface. Rather, the method involves aggregation, which can be detected by a variety of

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techniques, including but not limited to visual inspection, density scanning, light transmission, absorbance, color change and/or light scattering. Peptides, which can incorporate into pre-existing fibrils, are attached to colloids (*e.g.* gold colloids) or other particles (*e.g.* fluorescent beads). These peptide-presenting particles, along with free peptides, are mixed with solutions that contained fibrils (*e.g.* patient samples). As the particles (*e.g.* colloids) become incorporated into the fibril, they render the fibril optically detectable.

While it is not intended that the present invention be limited to the particular details of how this embodiment is formatted into an assay, a convenient approach is the 96-well ELISA plate format. For the reaction, the following reagents can be added to the ELISA plate in phosphate buffer (10mM phosphate, pH 7.4, 100 mM NaCl) and the following concentrations can be conveniently used: a synthetic A $\beta$  (1-40) peptide, with an N-terminal (His)<sub>6</sub> tag, at either 58.2  $\mu$ M or 14 $\mu$ M, 30 $\mu$ L NTA/Ni<sup>++</sup>-presenting gold colloids (preparation described below) and 0.6 $\mu$ M of A $\beta$ -amyloid fibril "seeds". The ELISA plate can then be incubated at 37°C, without agitation, for a desired period (*e.g.* 30 minutes to 2 hour). At 1 hour, large dark red aggregate structures are clearly visible in the wells that contained 58.2  $\mu$ M (His)<sub>6</sub>-A $\beta$  peptide. After a desired period (*e.g.* between 1 and 4.5 hours), the plate can be agitated briefly and then visually inspected. The aggregate structures are clearly visible in all wells that contained both (His)<sub>6</sub>-A $\beta$  peptide and A $\beta$  fibril "seeds". These aggregate, fibrillar-like structures are also able to form in solutions that contained up to 50% fetal bovine serum (FBS), albeit at a slower rate. When the concentration of FBS is serially diluted and incubated with fibril seeds and (His)<sub>6</sub>-A $\beta$  peptide at constant concentration, the rate of visible fibril formation increases with decreasing FBS concentration, which indicates that the assay was specific for A $\beta$  fibrils and not recognizing fibrillar species present in fetal serum. Wells that contained either fibrils or (His)<sub>6</sub>-A $\beta$  peptide, but not both, do not form visible aggregates. As a negative control, an irrelevant His-tagged peptide can be incubated with the colloids and seed fibrils. Such control solutions will not produce visible, fibrillar aggregates.

Using the above-described method, fibril seed concentrations as low as 10 picomolar are visibly distinguishable from solutions that contained colloids and A $\beta$  peptide, but did not contain seed fibrils.

The colorimetrics techniques described herein do not disrupt the assay process. A single

fluid mixture is prepared and, without any transfer or other agitation required, the mixture can be observed to determine aggregate formation or lack thereof. Alternatively, a particular assay can be exposed to agitation or other form of energy. For example, it may be desirable in certain instances to determine whether introduction of energy into a particular system affects aggregate formation. Energy can be introduced in the form of agitation such as stirring, shaking, vibrating, sonicating, or other mechanical agitation, exposure to electromagnetic radiation such as infrared, ultraviolet, or visible light, exposure to radio frequency energy, microwave radiation, or essentially any electromagnetic radiation at any portion of the spectrum. In some instances, exposure of a system to energy will affect the rate of aggregation which can be indicative of the potential affect of the energy on neurodegenerative disease processes.

The function and advantage of these and other embodiments of the present invention will be more fully understood from the examples below. The following examples are intended to illustrate the benefits of the present invention, but do not exemplify the full scope of the invention.

#### 15 Examples

The following examples serve to illustrate certain preferred embodiments aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents);  $\mu$  (micron); M (Molar);  $\mu$ M (micromolar); mM (millimolar); N (Normal); mol (moles); mmol (millimoles);  $\mu$ mol (micromoles); nmol (nanomoles); g (grams); mg (milligrams);  $\mu$ g (micrograms); ng (nanograms); L (liters); ml (milliliters);  $\mu$ l (microliters); cm (centimeters); mm (millimeters);  $\mu$ m (micrometers); nm (nanometers); nM (nanomolar);  $^{\circ}$ C (degrees Centigrade); PBS (phosphate buffered saline); U (units); d (days).

#### Example 1: Spectrophotometric Detection of Fibrils

In this example, 100  $\mu$ L aliquots that contained 14  $\mu$ M His-A $\beta$  (1-40) peptides and 30  $\mu$ L of NTA-thiol colloids were incubated with either 1  $\mu$ M of A $\beta$  fibril seeds, 50 pM A $\beta$ , fibril seeds or no fibrils. The mixtures were incubated at 37 $^{\circ}$ C for 0.5 hours then transferred to a cuvette and placed in a Hitachi U-2000 Spectrophotometer. For each aliquot, the absorbance at 569 nm was recorded, then the cuvette was removed, rapped sharply against a hard surface to accelerate peptide incorporation into the fibril, allowed to rest for 10 minutes then re-scanned at 569 nm.

We observed a sharp decrease in absorption at 569 (as the fibrils precipitated out of solution) for the solutions that contained fibril seeds, while those that contained only peptide remained stable (Figure 3). The height of the bars in the graph are the ratio of the initial measurement to the second measurement. 1) 1  $\mu$ M of A $\beta$  fibril seeds (striped bar) and no fibrils present (solid bar);  
5 and 2) 50pM A $\beta$  fibril seeds (striped bar) and no fibrils present (solid bar).

#### Example 2: Colloid Preparation

In this example, 1.5 ml of commercially available gold colloid (Auro Dye by Amersham) were pelleted by centrifugation in a microfuge on high for 10 minutes. The pellet was resuspended in 100  $\mu$ L of the storage buffer (sodium citrate and tween-20). 100  $\mu$ L of a dimethyl  
10 formamide (DMF) solution containing 40  $\mu$ M nitrilo tri-acetic acid (NTA)-thiol, 100  $\mu$ M ferrocene-thiol, and 500  $\mu$ M carboxy-terminated thiol was added (the ferrocene signaling entity is optional). Following a 3-hour incubation in the thiol solution, the colloids were pelleted and the supernatant discarded. They were then incubated in 100  $\mu$ L of 400  $\mu$ M tri-ethylene glycol-terminated thiol in DMF for 2 minutes at 55°C, 2 minutes at 37°C, 1 minute at 55°C, 2 minutes  
15 at 37°C, then room temperature for 10 minutes. The colloids were then pelleted and 100  $\mu$ L 100mM NaCl phosphate buffer were added. The colloids were then diluted 1:1 with 180  $\mu$ M NiSO<sub>4</sub> in the colloid storage buffer.

#### Example 3: Formation of a SAM-coded Electrode

In some of the embodiments described below, the examples involve SAM formation.  
20 collagen coating, cell growth, colloid formation, and Alternating Current Voltammetry (ACV). For SAM formation, glass microscope slides were sputtered with a layer of Ti followed by a layer of Au. Each electrode was incubated at RT for 0.5 hours with 300  $\mu$ L of a DMF solution that contained 10% methyl-terminated thiol (HS-(CH<sub>2</sub>)<sub>15</sub> CH<sub>3</sub>), 40% tri-ethylene glycol-terminated thiol, HS(CH<sub>2</sub>)<sub>11</sub>(CH<sub>2</sub>CH<sub>2</sub>)<sub>3</sub>OH, (formula) and 50% poly (ethynylphenyl) thiol  
25 (C<sub>16</sub>H<sub>10</sub>S). 2 ml of 400  $\mu$ M tri-ethylene glycol-terminated thiol were then added to a scintillation vial containing the chip and the vial was heat cycled in a water bath as follows: 2 minutes @ 55°C; 2 minutes @ 37°C; 1 minute @ 55°C; 2 minutes @ 37°C then RT for 10 min. Electrodes were then dipped in EtOH, then sterile PBS to rinse.

#### Example 4: Assay Demonstrating Extremely Sensitive Detection of Aggregates



We can sensitively detect pre-formed aggregates, characteristic of neurodegenerative disease, by monitoring color change & visible fibril formation as a function of time.

Gold colloids were derivatized with nitrilo tri-acetic acid/nickel self-assembled monolayers (NTA-Ni-SAMs), for the capture of histidine-tagged proteins, as described in Example 2. Colloids were coated with a low density of NTA-Ni (40 $\mu$ M NTA-thiol in a total thiol concentration of 1000:M) to inhibit the aggregation of nearest neighbor peptides, immobilized on a common colloidal particle. 30 $\mu$ L aliquots of the derivatized colloids were placed in wells of a 96-well plate. Histidine-tagged  $\beta$ -amyloid peptides (amino acids 1-40) were dissolved in phosphate buffer pH 7.4, then added to the colloid solutions such that the final concentration in a 100 $\mu$ L volume was 14 $\mu$ M.

Pre-formed fibrils, made from synthetic non-histidine-tagged  $\mu$ -amyloid peptides, were serially diluted and added to the solutions such that the final concentration of fibrils varied from 330 nanomolar to 50 picomolar. For every assay (each well), a negative control assay, in which no fibril was added, was performed (Figure 4). Solutions were incubated at 37° C for 1 hour and care was taken not to agitate the solutions. The color of the solutions that contained fibrils, changed from the characteristic pink color of gold colloids, to a deep purple/gray. Solutions that contained histidine-tagged  $\mu$ -amyloid (1-40), but no pre-formed fibrils changed to a lighter purple color, and at a slower rate than solutions to which fibrils were added. Dark masses, in the centers of wells that contained pre-formed fibrils, were clearly visible with the unaided eye (Figure 5). Observation using 40-fold magnification with a dissecting microscope revealed large, purple reticulums. These aggregates were clearly suspended in the fluid medium rather than sedimented onto the bottom of the plate. Negative control wells showed a grainy precipitate, but no large aggregates. Visual examination allowed us to discriminate between wells that contained pre-formed fibril and those that did not, down to an added fibril concentration of 50 picomolar (Figure 6). Results were recorded by photographs taken with a Nikon camera (ASA 800 film) attached to an inverted microscope at 40-fold magnification.

#### Example 5: Visualization Using Colloids

At higher peptide and fibril concentration, large colloid-decorated fibril structures are visible. Colloids bearing 40 $\mu$ M NTA-Ni SAMs were prepared as described in Example 2. 30 $\mu$ L aliquots of colloids were added to wells of a 96-well plate. Histidine-tagged  $\beta$ -amyloid peptide (1-40) was added to achieve a final concentration of 20 $\mu$ M in a final volume of 100 $\mu$ L. Pre-formed  $\beta$ -amyloid fibrils were added such that the final concentration was 20 $\mu$ M in a final volume of 100 $\mu$ M. Pre-

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formed  $\beta$ -amyloid fibrils were added such that the final concentration was  $1\mu\text{M}$ . The sample was incubated at 37 degrees C for 20 minutes then rapped sharply three times to accelerate aggregation.

After 1 hour continued incubation, at 37 degrees C, large colloid-fibril structures were clearly visible with the naked eye (Figure 7A). As colloids agglomerated onto the fibrillar structure, the surrounding solution cleared (no pink color). As a negative control, instead of a binding species fastened to the colloids, an irrelevant protein was fastened. The results are shown in Figure 7B.

#### Example 6: Drug Screening

We determined conditions under which histidine-tagged  $\beta$ -amyloid (1-40) peptides would form fibrils. Drug candidates from the Sigma Aldrich RBI drug library were separately added to each assay solution to determine which drug candidates inhibited fibril formation characteristic of Alzheimer's disease. Colloids bearing NTA-Ni-SAMs (previously described) were added as a method to visualize results.

To each well of a 96-well plate were added:  $30\mu\text{L}$  of NTA-SAM bearing colloids,  $65\mu\text{L}$  of a  $10.7\mu\text{M}$  solution of histidine-tagged  $\beta$ -amyloid (1-40) peptide ( $6.6\mu\text{M}$  final concentration), plus  $5\mu\text{L}$  of a drug candidate such that the final concentration of each drug would be between  $100$ - $200\mu\text{M}$ . Plates containing solutions were incubated at 37 degrees C for several hours. During this time, plates were visually examined and photos were taken to record progress periodically.

#### RESULTS:

Color change (from pink to blue) predicted which drugs inhibited fibril formation. At about 2 hours incubation time, one could clearly see with the unaided eye which wells contained drugs that had inhibited fibril formation by comparing the color of the solution to wells that contained positive control, i.e., no drug added and negative control, drug added but the histidine-tagged peptide was GST, not  $\beta$ -amyloid.

Colloids agglomerated onto fibrils in the wells also enabling the identification of wells that contained drugs that inhibited fibril formation by noting the lack of visible aggregates in the wells. 40-fold magnification enhanced the ability to discriminate between the action of drugs in different wells.

Figure 8 is a photocopy of a photograph of a small molecule library, rack 1 after 72 hours incubating at 37 degrees C. The wells on the right-most side of the plate (column 12) are negative control wells that remained pink, in which His-tagged GST replaced the  $\beta$ -amyloid peptide. The

next column to the left, column number 11 was the positive control that contained His-tagged  $\beta$ -amyloid and no drug candidate. Well G9 remained bright pink, indicating that the compound within that well inhibited fibril formation. Note wells A4, B5, C5, D5, F5, F9, G5, and H10 all displaying readily visibly-identifiable aggregate formation.

Monitoring color and or aggregate formation as a function of time under physiologic conditions are indicators of drug efficacy and drug stability.

Example 7: Using peptides free in solution to amplify

Colloids presenting NTA-Ni-SAMs were prepared as described in example 2; 40 $\mu$ M NTA-thiol in a total thiol concentration of 1000 $\mu$ M was used. Histidine-tagged  $\beta$ -amyloid (1-40) peptides were added to achieve a final concentration of 58.2 $\mu$ M in 100 $\mu$ L final volume. As a negative control, an irrelevant histidine-tagged peptide, GST, was added to colloid solutions in place of the  $\beta$ -amyloid peptide. Solutions were incubated at 37 degrees C. Within 15 minutes large aggregates were clearly visible in solutions that contained histidine-tagged  $\beta$ -amyloid peptide. No structures were visible in control solutions. Solutions with  $\beta$ -amyloid peptide turned gray/purple while negative control solutions remained pink. These results are consistent with the idea that free  $\beta$ -amyloid peptides in solution became aggregated and acted to amplify or accelerate the aggregation process.

Example 8: Electronic detection

Our strategy to electronically detect  $\beta$ -amyloid fibrils was to first incorporate histidine-tagged  $\beta$ -amyloid peptides (1-40) into pre-formed fibrils made from  $\mu$ -amyloid 1-42 peptides. Colloids bearing NTA and a ferrocene derivative, for electronic signaling, were added so that at least some of the His-tagged  $\beta$ -amyloid peptides were attached to the colloidal particles. Our aim was to then add magnetic beads that bore binding ligands that would bind to the colloid-decorated fibrils and magnetically recruit them to the working electrode.

Colloids bearing NTA-SAMs were prepared as described in Example 2 (with the exception that 100 $\mu$ M octamethyl ferrocene-thiol was used in place of a standard ferrocene-thiol). 30 $\mu$ L of colloids were added to each assay solution. To facilitate alternating current voltammetry (ACV) analysis, without having to pipet solutions after peptide addition (which would accelerate fibril formation of peptides in the absence of pre-formed fibrils), assay solutions were mixed in a 1ml. capacity silicon gasket clamped over a gold-coated glass slide that had been derivatized with a self-

assembled monolayer as described in Example 3, over a stationary magnet. Histidine-tagged  $\beta$ -amyloid peptide (amino acids 1-40) were added to a final concentration of 14 $\mu$ M in a 100 $\mu$ L volume. Pre-formed fibrils made up of  $\beta$ -amyloid peptide (1-42) were added to achieve a final concentration of 15 $\mu$ M. Negative control solutions were: (1) colloids, histidine-tagged GST plus fibrils, but no His-tagged  $\beta$ -amyloid peptide (1-40); (2) colloids, His-tagged  $\beta$ -amyloid peptide (1-40) but no fibrils; and (3) all the components expected to give a positive result, but measured at time zero, before His-tagged peptides could incorporate into the fibril. Solutions were incubated at 37 degrees C for 20 minutes. Commercially available magnetic beads (from Bang Labs and Prozyme) that were derivatized with Protein A (which binds the Fc portion of antibodies) were pre-bound to 1/10 their binding capacity with an antibody (purchased from Biosource Int.) that recognizes  $\beta$ -amyloid peptide 1-42, but not 1-40. 20 $\mu$ L of the antibody-presenting magnetic beads were injected into each solution immediately prior to ACV analysis (10 Hz.; 25mVolts overpotential; Ag/AgCl reference electrode with Pt auxiliary; SAM-coated gold chip acted as the working electrode).

#### RESULTS:

The solution that contained histidine-tagged  $\beta$ -myloid peptides (1-40), colloids bearing NTA-Ni and ferrocene plus pre-formed fibrils, *that had been incubated at 37 degrees C for 20 minutes*, produced a current peak of 2.0  $\mu$ Amps at the characteristic oxidation potential (220 mVolts) of the ferrocene derivative (octamethyl ferrocene) that was attached to the colloidal particle, see Figure 9, trace A.

An identical solution that was measured immediately, before histidine-tagged peptides could incorporate into the fibril, produced an insignificant peak of approximately 0.17  $\mu$ Amps at 220 mVolts, Figure 9, trace B.

Another negative control solution that contained all the components, but no pre-formed fibril generated an insignificant peak of .022  $\mu$ Amps (Figure 9, trace C).

A third negative control solution that contained pre-formed fibrils but in which histidine-tagged GST protein was attached to the colloids in place of histidine-tagged  $\beta$ -amyloid peptides produced no current peak (Figure 9, trace D).

#### Example 9: Light Scattering Analysis

A commercially available light scattering device, that quantitates the average diameter of particles in solution, was used to analyze solutions that contained NTA-Ni-SAM coated colloids and

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histidine-tagged  $\beta$ -amyloid (1-40) peptides in the presence or absence of pre-formed fibril. Colloids were prepared as described in Example 2, histidine-tagged  $\beta$ -amyloid was added such that the final concentration was 10.2 $\mu$ M, in a final volume of 100 $\mu$ L. Pre-formed fibrils were added such that the final concentration was 2 $\mu$ M. Baseline measurements, of average particle diameter were taken of the reagents alone to ensure that none of the components were aggregating in the absence of  $\beta$ -amyloid fibrils.

#### Baseline Measurements:

1. 40 $\mu$ M NTA-SAM-derivatized colloids alone; diameter = 14.87 nm
2. colloids presenting histidine-tagged A $\beta$  1-40 peptides; diameter = 14.96 nm
3. colloids (without surface-immobilized His-tagged A $\beta$  1-40) mixed with 1 $\mu$ M  $\beta$ -amyloid peptide (1-42) pre-formed fibrils; diameter = 15.93 nm; t=0

#### Experiment:

1. colloids presenting histidine-tagged A $\beta$  1-40 peptides, mixed with 1 $\mu$ M  $\beta$ -amyloid peptide (1-42) pre-formed fibrils, measured at t=0 15.5 nm.
2. colloids presenting histidine-tagged A $\beta$  1-40 peptides, mixed with 1 $\mu$ M  $\beta$ -amyloid peptide (1-42) pre-formed fibrils, measured at t=1.5 hours, under high intensity lamp: diameter = greater than 10,000 nm, see Figure 10.

#### Negative controls:

3. colloids presenting histidine-tagged A $\beta$  1-40 peptides; buffer was added in place of the pre-formed fibrils; measured at t=0; diameter = 14.96 nm.
4. colloids presenting histidine-tagged A $\beta$  1-40 peptides; buffer was added in place of the pre-formed fibrils; measured at t=1.5; diameter = 579.7 nm, see Figure 11.

These results show that the amount of aggregate in a specimen can be quantitated, by measuring the diameter of resultant macro-structures. A light scattering device, that simultaneously measures the average particle diameter of solutions in 96-well plate format, is commercially available. Until now, attempts to use light scattering to distinguish between peptide monomers and  $\beta$ -amyloid aggregates have met with limited success because the diameters of both species are very small and at the lower limits of detection ( $\beta$ -amyloid fibrils are on the order of hundreds of nanometers), and the difference between monomers and aggregates is also small. However, the

inventor illustrates that ligand-bearing colloids act to link together smaller, hard to detect fibrils, and stitch them into easily detectable macro-structures.

#### Example 10: Specificity

To determine whether the  $\beta$ -amyloid peptides presented by the derivatized colloids would incorporate non-specifically into fibrillar species that would be present in a normal diagnostic sample, we performed the visual assay in fetal bovine serum (FBS). (FBS contains 100-fold more irrelevant protein than CSF, including other fibrillar species.) 1  $\mu$ M A $\beta$  1-42 fibrils were added to 30  $\mu$ L of 40  $\mu$ M NTA-SAM colloids that presented histidine-tagged A $\beta$  1-40 in solutions of varying FBS concentration. The agglomeration of derivatized colloids onto fibrils did not increase with increasing FBS concentration, indicating that the assay was not prone to artifacts caused by other proteins or fibrillar species. Negative control solutions, which contained His-tagged A $\beta$  1-40 immobilized on colloids but did not contain pre-formed A $\beta$  fibrils, remained pink; no structures were observed under 40- or 100-fold magnification.

#### Example 11: Using the Extent of Visible Fibrillar Structure to Diagnose Alzheimer's Disease in CSF Samples

In a blinded study using CSF samples (unknown to the operator if one, both or neither came from AD patients), 30  $\mu$ M aliquots of colloids bearing 40  $\mu$ M NTA-SAMs, prepared as described in example 2, were added to wells of a 96-well plate. His-tagged A $\beta$  (1-40) peptides were added to achieve a final concentration of 3.75  $\mu$ M. Aliquots of CSF from two Alzheimer's disease patients were added to the colloid/peptide solutions. The amount of CSF added was varied to give final CSF concentrations of either 12.5% or 25%. For each CSF sample tested, conditions were varied as follows: (1) sample solutions were doped or "seeded" with pre-formed A $\beta$  fibrils (1  $\mu$ M; 1-42); (2) solutions were seeded with pre-formed A $\beta$  fibrils (0.1  $\mu$ M; 1-42); (3) solutions were not seeded with anything; and (4) as a negative control no probe His-A $\beta$  was immobilized on the colloids. After 3 hours at 37°C, fibrillar macro-structures were visible in all of the test solutions, except for the negative control, that contained CSF from patient #101. The extent of the fibrillar structures increased with increasing CSF concentration. Similarly, structure formation also increased with increasing seed concentration. The other CSF-containing solutions had turned from pink to purple but did not show any fibril structure formation under 40-fold magnification. Control solutions (no immobilized His-A $\beta$  on colloids) remained pink and did not show any signs of structure formation.

After overnight incubation (10 hours total), fibrils were also clearly visible in solutions that contained CSF from patient #109, although they were still much less extensive than those from the first patient. Like patient one, the extent of structure formation increased with increasing CSF concentration and with increasing  $\beta$ -amyloid seed concentration. Examination of sample records confirmed the CSF samples were both from Alzheimer's patients and that disease progression in the first patient (101), based on time since diagnosis and the Blessed Dementia Scale score, was about twice that of the second patient. The Blessed dementia scale score for patient 101 was 21 while the score for patient 109 was 12.  $\beta$ -amyloid concentration in patient 101 had been measured at 8 mgs/ml while # 109 was 4.6 mgs/ml.

#### Example 12: Detection and Diagnosing of CJD

In this hypothetical example, colloids presenting NTA-Ni are added to a solution containing a His-tagged PrP protein in its soluble (unconverted form) such that at least some of the His-tagged peptides are attached to the colloids. A sample suspected of containing infectious PrP, is added. The infectious PrP will convert (induce aggregation that includes a radical conformational change from alpha helix to pleated beta sheet that is, unlike normal PrP, resistant to Proteinase K digestion) the normal PrP protein, both on colloids and free in solution, such that they can then convert other PrP proteins. This sort of signal amplification, or conversion amplification, induces massive aggregation that can be readily detected as a colorimetric (pink to purple) change and the generation of large protein aggregates that can be detected with the unaided eye or by light scattering devices. Note that for this signal amplification to occur through the conversion of added normal PrP, the added PrP (His-tagged or not) must be derived from the same species as the PrP that is expected in the sample, or from a species whose PrP can be converted by the infectious sample.

Alternatively, one may desire an assay that is quantitative and does not include the signal amplification described above or an assay can detect prion diseases of unknown species origin. In this case, a peptide or protein derived from the prion protein (PrP), such as sequences homologous to 119-141 (GAVVGGLG GYMLGSAMSRPMMHF) which was excised from Syrian hamster PrP would be histidine-tagged, preferably at the N-terminus and added to colloids presenting NTA-SAMs as described in Example 2. This sequence has been shown to bind to multiple sites on converted or infectious PrP and thus inhibit its ability to "convert" other

normal PrP proteins. A sample suspected of containing infectious PrP, would be added to the colloids and peptides.

If the sample contained infectious PrP, aggregation of colloid-attached peptides would occur and could be detected as described above. These are sequences derived from hamster PrP that in addition to full length protein could be his-tagged and used in the assay.

106-128 (KTNMKHMAGAAAAGAVVGGLGGY)

109-141 (MKHMAGAAAAGAVVGGLGGYMLGSAMSRPMMHF)

113-141 (AGAAAAGAVVGGLGGYMLGSAMSRPMMHF)

119-141 (GAVVGGLGGYMLGSAMSRPMMHF)

117-141 (AAGAVVGGLGGYMLGSAMSRPMMHF)

115-141 (AAAAGAVVGGLGGYMLGSAMSRPMMHF)

113-141 (AGAAAAGAVVGGLGGYMLGSAMSRPMMHF)

Chabry J, Caughey B, Chesebro B, J Biol Chem 1998 May 22; 273(21):

13203-7; Residues 90-145 of Syrian hamster PrP, J. Mol. Biol. (1997) 270, 574-586.

#### 15 Example 13: Electronic Detection

In this hypothetical example, the presence of prion disease in a sample can also be electronically detected. Colloids presenting NTA-Ni and octamethyl ferrocene are added to a solution containing a His-tagged PrP protein in its soluble (unconverted form) such that at least some of the His-tagged peptides are attached to the colloids. Magnetic particles are also added to the assay that either present PrP or an antibody against it. A sample suspected of containing infectious PrP, is added. The infectious PrP will convert the normal PrP both on the colloid and free in solution, causing them to also participate in converting other proteins which results in massive aggregation. Massive aggregation results which incorporates both magnetic particles and colloidal particles into the same aggregates, which are then magnetically drawn to an electrode that can be SAM-coated and analyzed by ACV.

#### 25 Example 14: ELISA

A technique familiar to those skilled in molecular or cellular biology is used. Enzyme-linked Immunosorbent Assays (ELISA) (*Current Protocols in Molecular Biology, Volume 2, Immunology 11.2*, 1996, copyright from John Wiley and Sons Inc. 1994-1998). Normally when one performs an ELISA, a first species is directly or indirectly attached to a plastic substrate. A



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second species is added, the plate is washed and the presence of the second species is detected by binding to it a "secondary" antibody that also has a signaling capability; the secondary antibody is usually conjugated to an enzyme, typically horseradish peroxidase (HRPO), alkaline phosphatase (AP), or a fluorescent tag which is capable of performing a reaction on an added substrate that results in a color change (detected by a spectrophotometer), or a fluorescence labeling tag that can be detected by a fluorimeter (see, for e.g., "Localization of a passively transferred human recombinant monoclonal antibody to herpes simplex virus glycoprotein D to infected nerve fibers and sensory neurons in vivo", Sanna PP, Deerinck TJ, and Ellisman MH, 1999, Journal of Virology Oct. Vol 73 (10 8817-23)). Most often, for convenience, so that every antibody does not need to be conjugated to an enzyme, a mouse antibody is used as the specific recognition antibody, then an enzyme-conjugated rabbit-anti-mouse antibody is added.

Using the technology described herein, one can greatly increase the sensitivity of ELISAs and detect the presence of the immobilized target species using a natural ligand (protein or peptide) or a drug candidate as the probe molecule. The presence of the immobilized species is detected by binding to it a *ligand* attached to a colloid that also presents a plurality of horseradish peroxidase (HRPO) or alkaline phosphatase (AP). The enzyme can be conveniently linked to the colloid by a variety of means, including a histidine-tag attached directly to the enzyme, or by binding a mouse-anti-goat enzyme- conjugated antibody to a goat antibody that is attached to the colloid via a histidine-tagged protein G (Akerstrom, B., Nielson, E., Bjorck, L. Journal of Biological Chemistry, 1987 Oct. 5 Vol. 262 (28); pgs. 13388-91 and Fahnestock, S.R., Alexander, P., Nagle, J. and Filpula, D. (1986) Journal of Bacteriology Vol. 167, 870-880). By binding a ligand co-immobilized on a colloid with a plurality of enzymes, to the target species *in place of* a secondary antibody, the ratio of signaling molecules to binding events is increased by orders of magnitude. The binding of one antibody or ligand on the colloid to a presented antigen on an ELISA plate indirectly results in the binding of thousands or millions of enzymes. Alternatively, a known species can purposely be attached to wells of a 96-well plate so that one can probe with colloids that each present a separate drug candidate along with the signaling enzymes. Currently, it is not possible to do this with existing ELISA technology each drug candidate can not be conjugated to an enzyme. Alternatively, the natural ligand for the immobilized target can be presented on the colloid along with the signaling enzymes and drug

candidates added to each well of the plate to disrupt the interaction. Unbound colloids and thus their signal are lost in a wash step. The target for the antibody- or ligand-presenting colloids can be a cell or a protein bound directly or indirectly (via another antibody or ligand) to an ELISA plate. The advantage to this modification of an ELISA is not only sensitivity, but also efficiency. Because several hundred signaling enzymes remain bound via the colloids to one antigen, substrate hydrolysis will occur more quickly, and less time will be needed for an adequate reading.

In this hypothetical example, it is described how one would detect the presence of aggregate-forming or fibril-forming, proto fibril, or aggregate species in a sample, or screening for drugs that would inhibit neurodegenerative disease, on an ELISA plate. A binding species that binds a neurodegenerative disease aggregate or fibril-forming species, or an antibody to a fibril or aggregate-forming species, proto fibril or aggregate (specifically, an antibody to A $\beta$ 1-42) is immobilized in a well of an ELISA plate. In a diagnostic assay, the antibody then is exposed to a sample suspected of containing an aggregate-forming or fibril-forming species, and mixed with the sample is a binding species carrying a metal-binding tag (histidine-tag) optionally amplifier species, as described above, and colloid particles with a chelate coordinating a metal (NTA) and a non-electronic signaling entity each fastened to the particle. The non-electronic signaling entity could be a fluorescent tag, horse radish peroxidase, alkaline phosphatase, or the like.

Where one wants to screen for drugs using this assay, the same procedure is carried out but in the presence of a candidate drug for inhibiting neurodegenerative disease.

Example 15: Cell-Based Screening Assay for Candidate Drugs for Inhibition of Neurodegenerative Disease at a Variety of Stages of Biochemical Progression of the Disease

In this hypothetical example, it is described how one would test a candidate drug for activity against cellular processes involved in production of neurodegenerative disease aggregate-forming or fibril-forming species such as the enzymes  $\beta$ -secretase and the  $\gamma$ -secretase. (Wolfe, et al., *Nature*, 1999 April 8; 398 (6727):513-7; Haass, *Nat. Med.* December, 1999; 1 (12):1291-6).

In a well of an ELISA plate, a cell is placed which can secrete a fibril-forming or aggregate-forming species, specifically, the A $\beta$ -fibril-forming species. The cell is transfected

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with an over-expression plasmid for A $\beta$  (LaBlanc, et al., *J Neurosci Res.*, April, 1992, 31(4):635-45). After a period of time sufficient to allow the cell to overexpress the A $\beta$  fibril-forming species, into the well is introduced components of one of the above-described assays for drug screening. For example, colloids and binding species fastened to or adapted to be fastened to the colloids and, optionally, amplifier species are introduced into the well. The ability of the drug to inhibit A $\beta$  fibril-forming species (which drug is introduced prior to overexpression begins) is monitored by observing relative agglomeration of colloids in the well, indicative of the aggregation potential of the secreted substance.

This example represents yet another aspect of the invention involving exposing a candidate drug for inhibition of neurodegenerative disease to a cell adapted to secrete neurodegenerative disease fibril- or aggregate-forming species, and determining the effect of the candidate drug on the aggregation potential of material secreted from the cell.

Those skilled in the art would readily appreciate that all parameters listed herein are meant to be exemplary and that actual parameters will depend upon the specific application for which the methods and apparatus of the present invention are used. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described.

What is claimed is:

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1. A kit comprising:

a first article having a surface;

a second article having a surface; and

a plurality of binding species capable of binding a neurodegenerative disease aggregate-  
5 forming or fibril-forming species, at least some of which binding species are fastened to or  
adapted to be fastened to the surface of the first article and at least some of which binding  
species are fastened to or adapted to be fastened to the surface of the second article.

2. A composition comprising:

10 a binding species capable of binding a neurodegenerative disease aggregate-forming or  
fibril-forming species; and

an electronic signaling entity immobilized relative to the binding species.

3. An article comprising:

15 a surface of the article;

a binding species capable of binding a neurodegenerative disease aggregate-forming or  
fibril-forming species fastened to the surface; and

a signaling entity fastened to the surface.

20 4. A kit comprising:

an article having a surface, which article is not an SPR article; and

a plurality of binding species capable of binding a neurodegenerative disease aggregate-  
forming or fibril-forming species, at least some of which binding species are fastened to or  
adapted to be fastened to the surface.

25 5. A kit comprising:

an article having a surface; and

a plurality of binding species capable of binding a neurodegenerative disease aggregate-  
forming or fibril-forming species, at least some of which binding species are fastened to or  
30 adapted to be fastened to the surface, wherein the surface has a chemical functionality

substantially inhibiting non-specific binding of aggregate-forming or fibril-forming species.

6. A system comprising a neurodegenerative disease fibril or aggregate, and at least two particles immobilized relative to the fibril or aggregate.

5

7. A system comprising at least two particles each immobilized relative to a neurodegenerative disease aggregate-forming or fibril-forming species.

8. A composition comprising:  
10 a binding species capable of binding a neurodegenerative disease aggregate-forming or fibril-forming species, fastened to a moiety that can coordinate a metal.

9. A method comprising:  
providing an article having a first surface and a plurality of binding species capable of  
15 binding a neurodegenerative disease aggregate-forming or fibril-forming species fastened to or adapted to be fastened to the surface; and  
exposing the first surface to a sample suspected of containing neurodegenerative disease aggregate-forming or fibril-forming species.

20 10. A method comprising:  
forming neurodegenerative disease aggregates or fibrils in a sample containing neurodegenerative disease aggregate-forming or fibril-forming species; and  
exposing the aggregates or fibrils to an article having a surface and a plurality of binding  
species capable of binding the aggregates, fibrils, aggregate-forming species, or fibril-forming  
25 species, the binding species fastened to or adapted to be fastened to a surface of the article.

11. A method comprising:  
providing a binding species fastened to or adapted to be fastened to a surface of a first  
article, which binding species is not a neurodegenerative disease aggregate-forming or fibril-  
30 forming species, but is capable of binding a neurodegenerative disease aggregate-forming or

fibril-forming species; and

converting the binding species into a neurodegenerative disease aggregate-forming or fibril-forming species.

5           12.     A method comprising:

providing a binding species that is not a neurodegenerative disease aggregate-forming or fibril-forming species but is capable of binding a neurodegenerative disease aggregate-forming or fibril-forming species;

allowing the binding species to interact with a neurodegenerative disease aggregate-

10   forming or fibril-forming species thereby being converted to a neurodegenerative disease aggregate-forming or fibril-forming species, and to participate in aggregation characteristic of the presence of neurodegenerative disease aggregate-forming or fibril-forming species; and

detecting the aggregation characteristic of the presence of the neurodegenerative disease aggregate-forming or fibril-forming species.

15

13.     A method comprising:

providing a binding species, capable of binding a neurodegenerative disease aggregate-forming or fibril-forming species, fastened to or adapted to be fastened to a surface of an article;

20           optionally allowing the binding species to fasten to the surface of the article;

allowing the binding species to bind a neurodegenerative disease aggregate-forming or fibril-forming species without conversion of the binding species to an aggregate-forming or fibril-forming species; and

allowing a second binding species, fastened to or adapted to be fastened to a surface of a  
25   second article, to bind the aggregate-forming or fibril-forming species.

14.     A kit, method, composition, system, or article as in any preceding claim, wherein the binding species is a peptide.

30           15.     A kit, method, composition, system, or article as in any of claims 1-5, wherein

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the binding species is a protein.

16. A kit, method, composition, system, or article as in any of claims 1-5, wherein the binding species is a sequence from a protein.

5

17. A kit, method, composition, system, or article as in any of claims 1-5, wherein the binding species is a small molecule.

18. A kit as in claim 17, wherein the small molecule is Congo red or Thioflavin-T.

10

19. A kit, method, composition, system, or article as in any of claims 1-5, wherein the binding species is an antibody to the aggregate-forming or fibril-forming species.

20. A kit, method, composition, system, or article as in claim 1, wherein the binding species is capable of binding neurodegenerative fibrils or aggregates.

15

21. A kit as in claim 20, wherein the binding species is capable of forming a macrostructure including a plurality of articles bound to a plurality of neurodegenerative disease fibrils or aggregates.

20

22. A kit, composition, article, system, or method as in any preceding claim, wherein the binding species is a protein capable of aggregation characteristic of neurodegenerative disease.

25

23. A kit, composition, article, system, or method as in any preceding claim, wherein the binding species is selected from among beta-amyloid proteins, amyloid proteins, Tau, synnuclein, PrP<sup>CJD</sup>, PrP<sup>BSE</sup>, PrP<sup>Scrapie</sup>, and fragments and fusions thereof.

24. A kit, composition, article, system, or method as in any preceding claim, wherein the protein, fragment or fusion is aggregate-binding, aggregate-formation-resistant.

30

25. A kit as in claims 1-5, wherein the article is a fluid-suspendible, isolatable particle.

26. A kit as in claim 25, wherein the article is a colloid particle.

26.1. A kit as in claim 26, wherein the article is a gold colloid particle.

27. A kit as in claims 1-5, wherein the article is an SPR chip.

28. A kit as in claim 22, further comprising particles fastened to or adapted to be fastened to at least some of the binding species.

29. A kit as in claims 1-5, further comprising particles fastened to or adapted to be fastened to at least some of the binding species.

30. A kit as in claims 1-5, wherein the article is a particle, the kit further comprising additional particles fastened to or adapted to be fastened to at least some of the binding species.

31. A kit as in claims 1-5, wherein the binding species fastened to or adapted to be fastened to the surface are fastenable to the surface via a metal binding tag/metal/chelate linkage.

32. A kit as in claim 31, wherein the surface carries a chelate coordinating a metal immobilized relative to the surface, and the binding species is derivatized with a polyamino acid tag.

33. A kit as in claim 31, wherein the surface carries a chelate coordinating a metal immobilized relative to the surface, and the binding species is derivatized with a histidine tag.

34. A kit as in claims 1-5, wherein the binding species fastened to or adapted to be fastened to the surface are fastenable to the surface via complementary nucleic acid sequence



pairs.

35. A kit as in claims 1-5, wherein the binding species carry a terminal cysteine and are fastened to the surface thereby.

5

36. A kit as in claim 30, comprising a plurality of particles fastened to at least some of the binding species, wherein the binding species are immobilized relative to the surface of the particles at a surface concentration small enough that, in the absence of auxiliary, non-surface immobilized aggregate-forming or fibril-forming species, particle agglomeration upon  
10 particle/particle exposure is hindered within a time frame allowing comparison of agglomeration in the absence of auxiliary aggregate or fibril-forming species with agglomeration in the presence of auxiliary aggregate or fibril-forming species.

37. A kit as in claim 30, further comprising a candidate drug for inhibiting the  
15 neurodegenerative disease.

38. A kit as in claims 3-5, wherein the surface carries a self-assembled monolayer.

39. A kit as in claim 38, wherein the self-assembled monolayer comprises a species  
20 that inhibits colloid/colloid self aggregation.

40. A kit as in claim 39, wherein the self-assembled monolayer contains charged moieties.

41. A kit as in claim 39, wherein the self-assembled monolayer contains carboxy-  
25 terminated species.

42. A kit as in claim 40, wherein the charged moieties include nitrilotriacetic acid, 2,2'-bis(salicylideneamino)-6,6'-demethyldiphenyl, or 1,8-bis(a-pyridyl)-3,6-dithiaoctane.

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43. A kit as in claim 42, wherein the charged moieties comprise nitrilotriacetic acid.

44. A kit as in claim 38, wherein the self-assembled monolayer comprises oligonucleotides.

5

45. A kit as in claim 44, wherein the self-assembled monolayer comprises DNA moieties.

10

46. A kit as in claim 38, wherein the self-assembled monolayer includes charged peptides.

47. A kit as in claim 38, the self-assembled monolayer deposited onto the surface from a solution including a surfactant.

15

48. A kit as in claim 638, the self-assembled monolayer deposited onto the surface from a solution including a carboxylate.

49. A kit as in claim 38, the self-assembled monolayer deposited onto the surface not during formation of the colloid particle itself.

20

50. A kit as in claim 38, the self-assembled monolayer deposited onto the surface in suspension in a fluid, the particle not present at a fluid-fluid interface.

25

51. A kit as in claim 38, wherein the self-assembled monolayer is a mixed self-assembled monolayer further comprising the binding species.

52. A kit as in claims 1 and 3-5, wherein the binding species is a peptide having a terminal cysteine and the surface is gold.

30

53. A kit, method, composition, system, or article as in any preceding claim, wherein

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the binding species is not a neurodegenerative disease aggregate-forming or fibril-forming species.

54. A kit, method, composition, system, or article as in claim 53, wherein the binding  
5 species is not capable of converting other binding species to neurodegenerative disease aggregate-forming or fibril-forming species.

55. A kit, method, composition, system, or article as in any preceding claim, wherein  
the binding species is converted by a neurodegenerative disease aggregate-forming or fibril-  
10 forming species to an aggregate-forming or fibril-forming species.

56. A kit, method, composition, system, or article as in claim 55, wherein the binding  
species converted to an aggregate-forming or fibril-forming species is able to convert other  
binding species to aggregate-forming or fibril-forming species.

15 57. A composition as in claim 8, wherein the moiety that can coordinate a metal comprises a polyamino acid tag.

58. A composition as in claim 8, wherein the moiety that can coordinate a metal  
20 comprises a histidine tag.

59. A composition as in claim 20, wherein the binding species is a peptide fastened at  
its N-terminus to the moiety that can coordinate a metal.

25 60. A kit as in claim 26, further comprising a moiety that can coordinate a metal immobilized relative to a surface of the article.

62. A kit as in claims 1-5, wherein the binding species is fastened to or adapted to be  
fastened to the surface via at least one of a chelate, carboxylate group, n-hydroxy-succinimide,  
30 nucleic acid sequence, glutathione, biotin, streptavidin, or fragment thereof.

63. An article as in claim 25, wherein the fluid-suspendable, isolatable particle is of no more than 250 nm cross section in any dimension.

64. A kit as in claim 25, wherein the fluid-suspendable, isolatable particle is of no more than 100 nm cross section in any dimension.

65. An article as in claim 60, comprising a colloid particle and a self-assembled monolayer on a surface of the particle comprising the moiety that can coordinate a metal.

66. An article as in claim 65, wherein the self-assembled monolayer includes a chelate.

67. An article as in claim 66, wherein the self-assembled monolayer includes nitrilotriacetic acid, 2,2'-bis(salicylideneamino)-6,6'-demethyldiphenyl, or 1,8-bis(a-pyridyl)-3,6-dithiaoctane.

68. An article as in claim 65, wherein the self-assembled monolayer is a mixed self-assembled monolayer including self-assembled monolayer-forming species, some but not all of the self-assembled monolayer-forming species including the moiety that can coordinate a metal.

68.1. An article as in claim 60, further comprising a signaling entity.

69. An article as in claim 68, comprising a plurality of signaling entities.

70. An article as in claim 68, comprising a plurality of signaling entities fastened to the colloid particle.

71. A system as in claim 7, each of the at least two particles fastened to a binding species binding the neurodegenerative disease aggregate-forming or fibril-forming species.

72. A system as in claim 6, wherein each of the at least two particles is immobilized relative to the fibril or aggregate via a binding species fastened to the particle and binding the fibril or aggregate.

5

73. A system as in claim 72, comprising a fibril or aggregate immobilized relative to at least two particles, at least one of the particles immobilized relative to a second fibril or aggregate.

10 74. A system as in claim 73, wherein the particles and fibrils or aggregates form a structure visible to the unaided human eye.

75. A method as in claim 10, comprising exposing the aggregates or fibrils to a plurality of colloids fastenable to the binding species, thereby linking the colloids to the fibrils or  
15 aggregates.

76. A method as in claim 75, comprising rendering the fibrils or aggregates visibly detectable via addition of the colloids.

20 77. A method as in claim 9, wherein the surface is a surface of a particle, the method comprising:

providing a plurality of particles carrying the binding species immobilized relative thereto; and

exposing the particles to the sample.

25

78. A method as in claim 77, further comprising determining the extent of agglomeration of the particles indicative of neurodegenerative disease aggregate-forming or fibril-forming species present in the sample.

30 79. A method as in claim 9, wherein the surface is a surface of a particle, the method

comprising:

providing a plurality of particles and binding species fastened to or adapted to be fastened to surfaces of the particles; and

exposing the particles and binding species to the sample.

5

80. A method as in claim 78, wherein the binding species and the sample are derived from diverse biological species.

80.1. A method as in claim 80, wherein the interacting species and sample are the same protein or protein fragments from diverse biological species.

10

81. A method as in claim 78, further comprising determining the extent of agglomeration of the particles.

15

82. A method as in claim 81, wherein the sample contains neurodegenerative disease aggregate-forming or fibril-forming species, the exposing step involving exposing the particles and binding species to the sample in the presence of a candidate drug suspected of inhibiting neurodegenerative disease fibril or aggregate formation.

20

83. A method as in claim 82, wherein the sample is secreted from a cell.

84. A method as in claim 83, further comprising determining the extent of agglomeration of the particles.

25

85. A method as in claim 84, comprising first exposing the cell that secretes the sample to a candidate drug suspected of inhibiting neurodegenerative disease, then exposing the particles and binding species to the sample.

30

86. A method as in claim 85, wherein the drug is suspected of inhibiting an enzyme whose activity has been linked to neurodegenerative disease.

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87. A method as in claim 86, wherein the candidate drug is suspected of inhibiting  $\beta$ -secretase.

88. A method as in claim 86, wherein the candidate drug is suspected of inhibiting  $\gamma$ -secretase.

89. A method as in claim 82, the exposing step involving exposing the particles to the sample in the presence of beta-amyloid including amino acid sequences of from 1-38 to 1-44.

90. A method as in claim 89, wherein the beta-amyloid peptide includes an amino acid sequence of 1-40.

91. A method as in claim 78, the exposing step comprising exposing the particles to a biological specimen drawn from a sample suspected of being associated with neurodegenerative disease.

100. A method as in claim 91, comprising:  
exposing the specimen to the particles and the binding species.

101. A method as in claim 91, wherein the sample comprises a blood sample.

102. A method as in claim 91, wherein the sample is drawn from a human patient.

103. A method as in claim 91, wherein the sample is drawn from an animal.

104. A method as in claim 91, wherein the sample is drawn from livestock.

105. A method as in claim 91, wherein the sample is drawn from livestock feed.

106. A method as in claim 91, wherein the sample is an organ donation sample.

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107. A method as in claim 78, further comprising observing a change in the sample visible to the eye upon exposure of the particles to the sample.

108. A method as in claim 107, wherein the visible change comprises the  
5 agglomeration of particles.

109. A method as in claim 108, wherein the visible change comprises the agglomeration of gold colloid particles.

110. A method as in claim 107, wherein the visible change comprises a color change.  
10

111. A method as in claim 78, further comprising measuring a change in effective particle/agglomerate size upon exposure of the particles to the sample using a light-scattering device.  
15

112. A method as in claim 78, comprising digitizing an image of the sample, then using pattern recognition to determine whether the sample contains aggregates.

113. A method as in claim 91, the binding species immobilized relative to the surface  
20 of the particles at a surface concentration small enough that, in the absence of auxiliary, non-surface immobilized aggregate-forming or fibril-forming species, particle agglomeration upon particle/particle exposure is hindered within a time frame allowing comparison of agglomeration in the absence of auxiliary aggregate-forming or fibril-forming species with agglomeration in the presence of auxiliary aggregate-forming or fibril-forming species.

114. A method as in claim 9, further comprising determining interaction of the binding species with any aggregate-forming or fibril-forming species present in the sample.  
25

115. A method as in claim 9, wherein the binding species and the sample are diverse  
30 biological species.



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116. A method as in claim 9, comprising exposing the surface to the sample in the presence of a binding species immobilized relative to a particle that binds to the aggregate-forming or fibril-forming species.

5 117. A method as in claim 116, wherein the particle carries an auxiliary signaling entity.

118. A method as in claim 117, wherein the auxiliary signaling entity comprises a dye, pigment, electroactive molecule, fluorescent moiety, up-regulating phosphor, or enzyme-  
10 fastened signaling moiety including horse radish peroxidase and alkaline phosphatase.

119. A method as in claim 116, wherein the surface is a surface of an electrode, and the particle carries an electroactive species immobilized relative to the surface.

15 120. A method as in claim 119, wherein the particle carries a plurality of immobilized electroactive species.

121. A method as in claim 120, wherein the plurality of electroactive species comprise metallocenes.

20

122. A method as in claim 120, wherein the plurality of electroactive species comprise ferrocenes.

25

123. A method as in claim 9, wherein the article is a magnetic bead.

124. A method as in claim 9, wherein the article is an SPR chip.

125. A method as in claim 9, wherein the article is an electrode.

30

126. A method as in claim 9, wherein the article is an ELISA plate.

127. A method as in claim 9, wherein the surface comprises a plurality of individually spatially-addressable regions.

128. A method as in claim 128, wherein the individually spatially-addressable regions  
5 comprise different wells of a multi-well plate.

131. A method as in claim 132, comprising exposing the sample to the surface in the presence of a candidate drug for inhibiting the neurodegenerative disease.

10 132. A method as in claim 133, further comprising observing a reduction in interaction of the aggregate-forming or fibril-forming species with the surface-immobilized binding species due to presence of the candidate drug.

135. A method as in claim 9, wherein the sample may or may not contain the  
15 aggregate-forming or fibril-forming species.

136. A method as in claim 145, wherein the sample is a drug-screening preparation that contains an aggregate-forming or fibril-forming species.

20 137. A method as in claim 9, wherein the surface is a surface of a particle, the method comprising:

forming a composition comprising, suspended in a fluid medium, the binding species, particles fastenable to the binding species, and magnetic beads fastenable to the binding species;  
and

25 exposing the composition to the sample.

138. A method as in claim 135, wherein the particles and/or the magnetic beads carry immobilized chelates, and at least some of the binding species carry a metal binding tag fastenable to a metal coordinated by the chelate.

-59-

139. A method as in claim 137, wherein at least some of each of the particles and beads are fastened to binding species.

140. A method as in claim 137, the composition further comprising a redox-active  
5 species fastened to or fastenable to the particles.

142. A method as in claim 137, further comprising exposing the composition to a sample suspected of containing a neurodegenerative disease aggregate-forming or fibril-forming species.

10 143. A method as in claim 137, further comprising exposing the composition to a sample containing a neurodegenerative disease aggregate-forming or fibril-forming species and a candidate drug for inhibition of the neurodegenerative disease.

15 144. A method as in claim 142, further comprising drawing at least some of the magnetic beads, fastened to aggregate-forming or fibril-forming species which are fastened to particles carrying redox-active agents, to an electrode and determining the presence of the redox-active agents proximate the electrodes.

20 145. A method as in claim 144, wherein the electrode is coated with an inhibitor of non-specific binding.

146. A method as in claim 145, wherein the inhibitor of non-specific binding comprises a self-assembled monolayer.

25 147. A method as in claim 146, wherein the self-assembled monolayer comprises a polyethylene glycol-terminated self-assembled monolayer-forming species.

149. A method as in claim 146, wherein the self-assembled monolayer further  
30 comprises a species that enhances permeability of the self-assembled monolayer to electrons.

-60-

150. A method as in claim 149, wherein the species enhancing permeability to electrons comprises a conductive self-assembled monolayer-forming species.

151. A method as in claim 149, wherein the species that enhances permeability to  
5 electrons comprises a species that causes defect sites in the self-assembled monolayer.

152. A composition, article, method, system, or kit as in any preceding claim, wherein the signaling entity is a multiple signaling entity.

10 153. A method as in claim 11, further comprising allowing the binding species to convert other binding species into neurodegenerative disease aggregate-forming or fibril-forming species.

154. A method as in claim 153, comprising allowing the binding species to interact  
15 with a neurodegenerative disease aggregate-forming or fibril-forming species thereby being converted in structure to a neurodegenerative disease aggregate-forming or fibril-forming species, then allowing the species to convert the other binding species.

155. A method as in claim 11, comprising exposing the system to non-surface bound  
20 auxiliary binding species that are not neurodegenerative disease aggregate-forming or fibril-forming species, and allowing the auxiliary binding species to be converted to neurodegenerative disease aggregate-forming or fibril-forming species.

156. A method as in claim 11, wherein the binding species initially is fastened to the  
25 surface of the article.

157. A method as in claim 11, wherein the binding species initially is not fastened to the surface of an article.

30 158. A method as in claim 13, wherein the binding species and the neurodegenerative

-61-

disease aggregate-forming or fibril-forming species are from different species of biological classification.

159. A method comprising:

5 forming a self-assembled monolayer on a surface by exposing the surface to a medium containing self-assembled monolayer-forming molecular species and surfactant.

160. A method comprising:

10 forming a self-assembled monolayer on a surface by exposing the surface to a medium containing self-assembled monolayer-forming molecular species and a carboxylate.

161. A method comprising:

15 forming a self-assembled monolayer on a surface of a colloid particle not during formation of the colloid particle itself.

162. A method comprising:

forming a self-assembled monolayer on a surface of a colloid particle in suspension in a fluid, the particle not present at a fluid-fluid interface.

20 163. A method as in claims 159-160, wherein the medium is a solution or suspension containing the self-assembled monolayer-forming molecular species and the surfactant.

164. A method as in claim 159 further comprising, after forming the self-assembled monolayer on the surface, removing any residual surfactant from the self-assembled monolayer.

25

170. A method comprising:

providing at least two binding species, each capable of binding a neurodegenerative disease aggregate-forming or fibril-forming species, immobilized relative to each other or adapted to be immobilized relative to each other, thereby defining a neurodegenerative disease aggregate or fibril linker;

30

-62-

exposing the linker to a sample suspected of containing neurodegenerative disease aggregate-forming or fibril-forming species or a solution containing a candidate drug for inhibiting the neurodegenerative disease.

5           171. A method comprising:  
          exposing a cell adapted to secrete a neurodegenerative disease aggregate- or fibril-forming species to a candidate drug for inhibition of neurodegenerative disease; and  
          monitoring the potential of materials secreted from the cell for formation of aggregates characteristic of neurodegenerative disease.

10           172. A method comprising:  
          forming a solution containing a species capable of binding neurodegenerative disease aggregate-forming or fibril-forming species and one of a sample suspected of containing neurodegenerative disease aggregate-forming or fibril-forming species for a candidate drug for  
15   inhibition of neurodegenerative disease aggregate or fibril formation; and  
          without transferring any components into the solution or removing the solution from its container, detecting aggregation in the solution characteristic of neurodegenerative disease.

20           173. A method comprising introducing energy into the fluid.

          174. A method as in any preceding claim, comprising rendering a fibril or aggregate visible.

          175. An article, method, kit, system, or composition as in any preceding claim,  
25   wherein the article is a fluid suspendable particle.

          176. An article, method, kit, system, or composition as in any preceding claim, wherein the article is a magnetically suspendable particle.

30           177. An article, method, kit, system, or composition as in any preceding claim,

wherein the article is a self-suspendable particle.

178. A method, article, system, kit, or composition as in any preceding claim, wherein the binding species is specifically fastened or adapted to be specifically fastened to the surface.

5

179. A method, article, system, kit, or composition as in any preceding claim, wherein the sample is a naturally-occurring sample.

10 180. A method, article, system, kit, or composition as in any preceding claim, wherein the sample is a structurally predetermined sample.

Figure 1

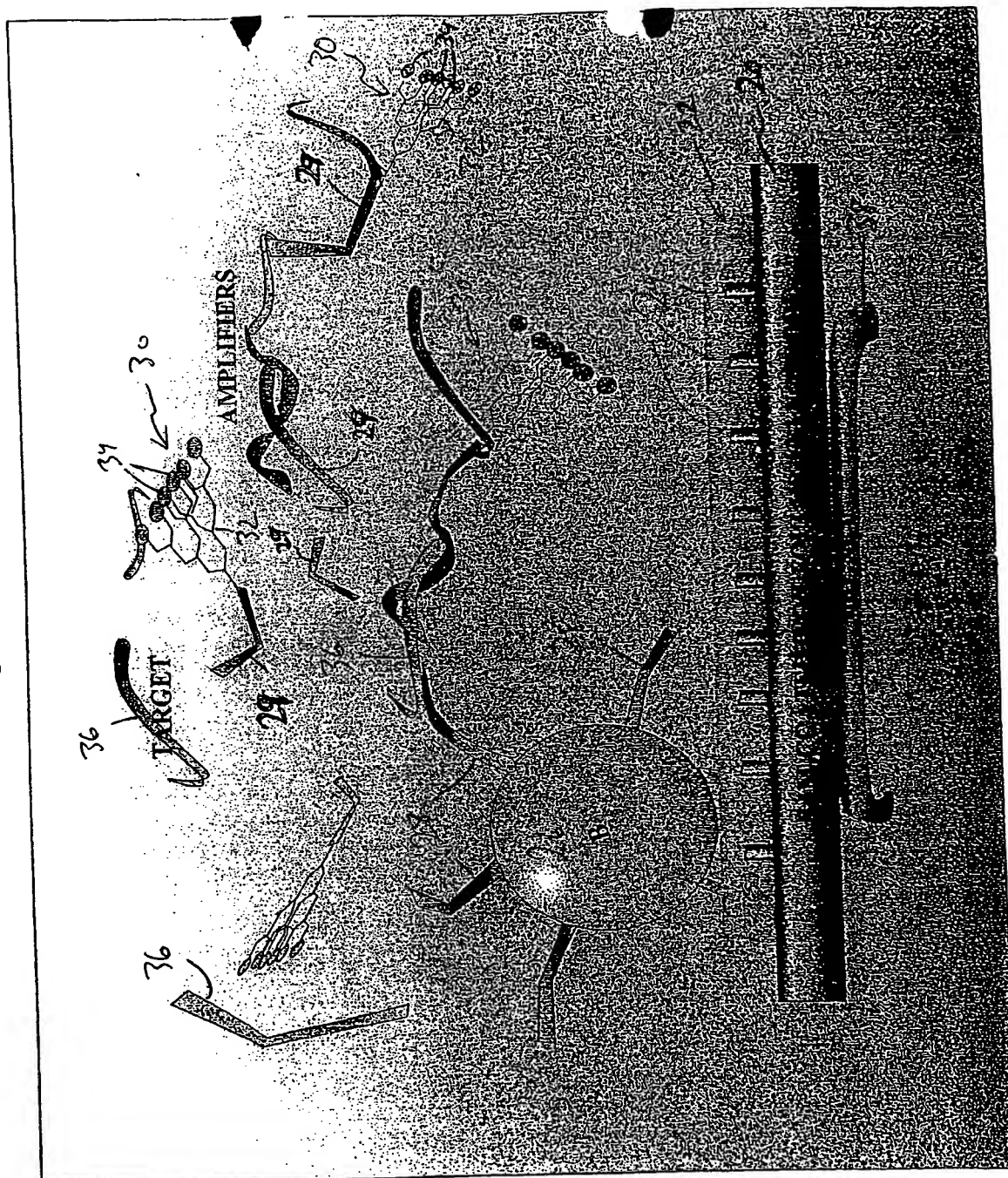
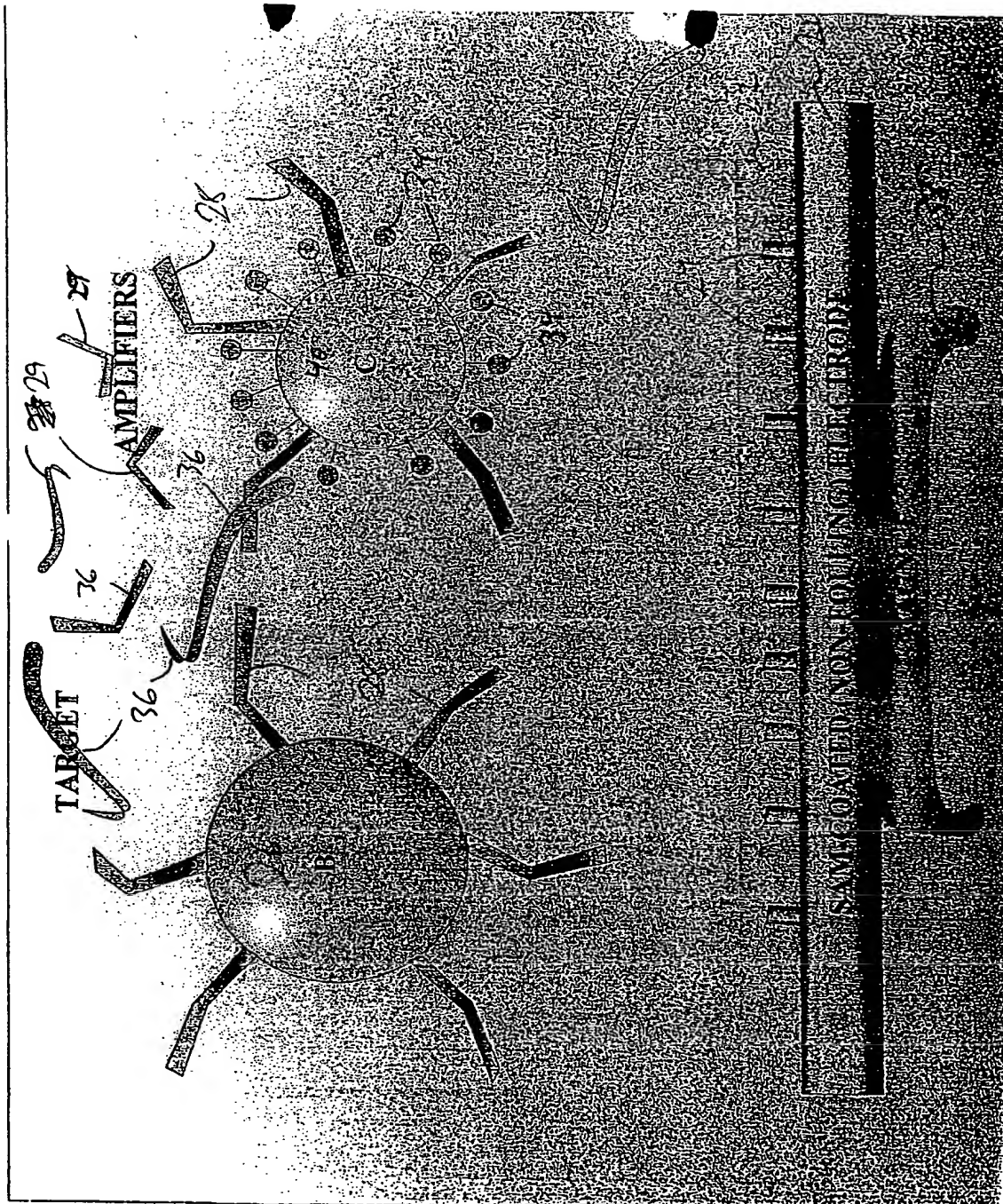
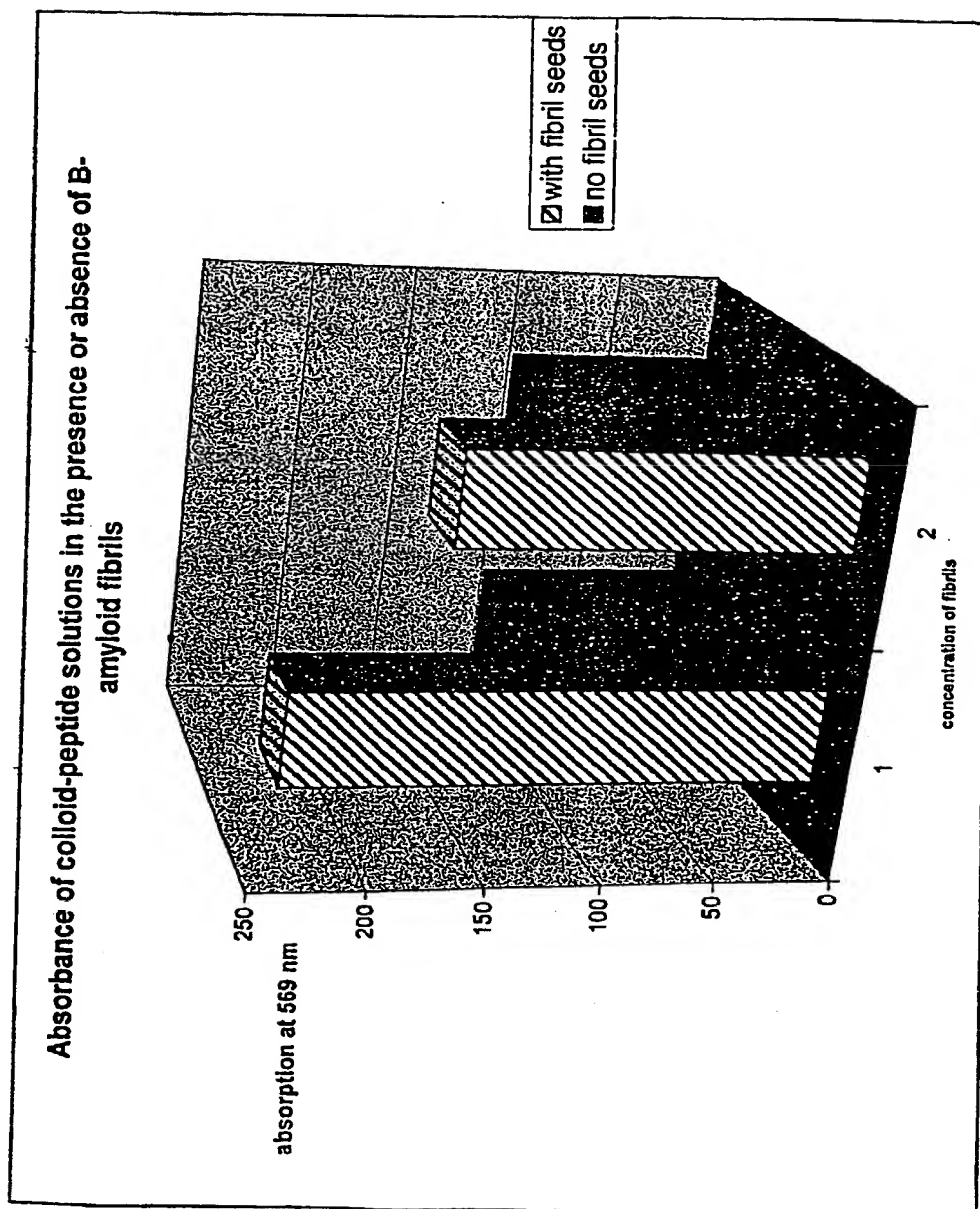




Figure 2



Fig. 3

Negative control, no  $\beta$ -Amyloid fibril

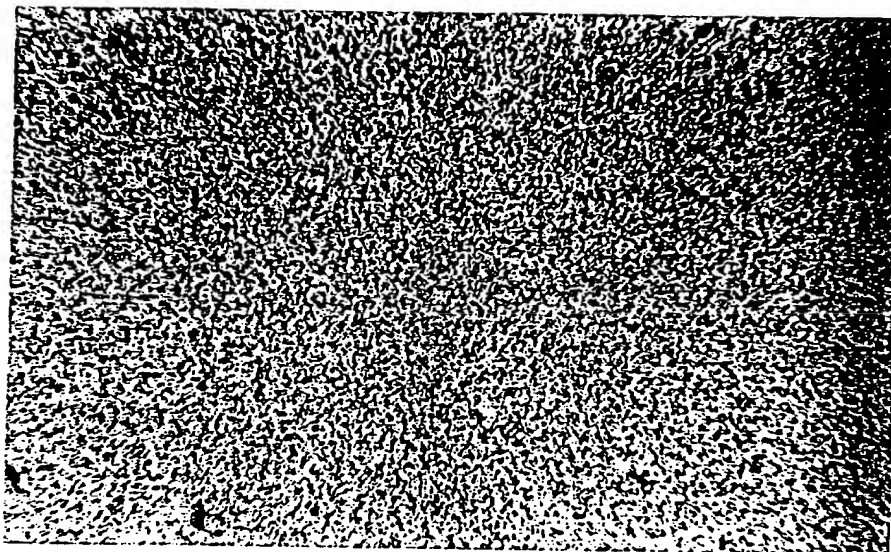


FIG. 4

300nM  $\beta$ -Amyloid fibril



FIG. 5

50pM  $\beta$ -Amyloid fibril

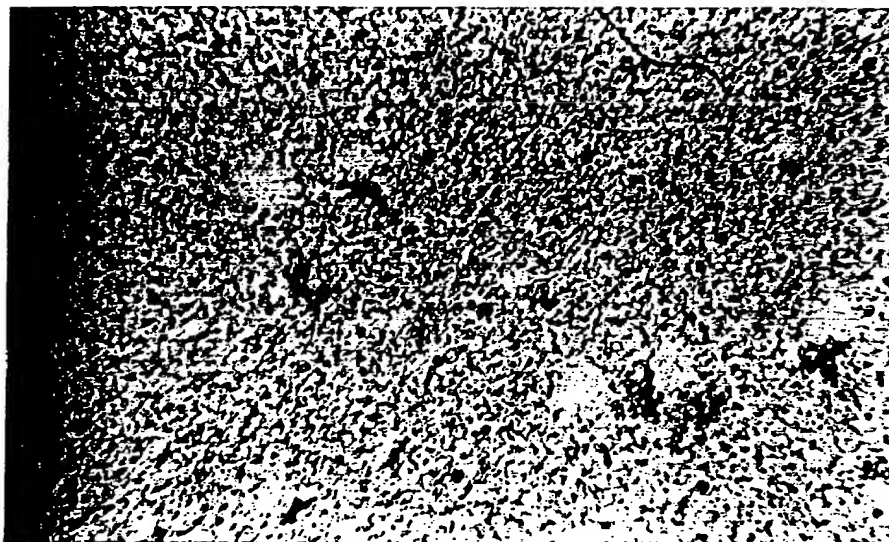


FIG. 6



Fig. 7A

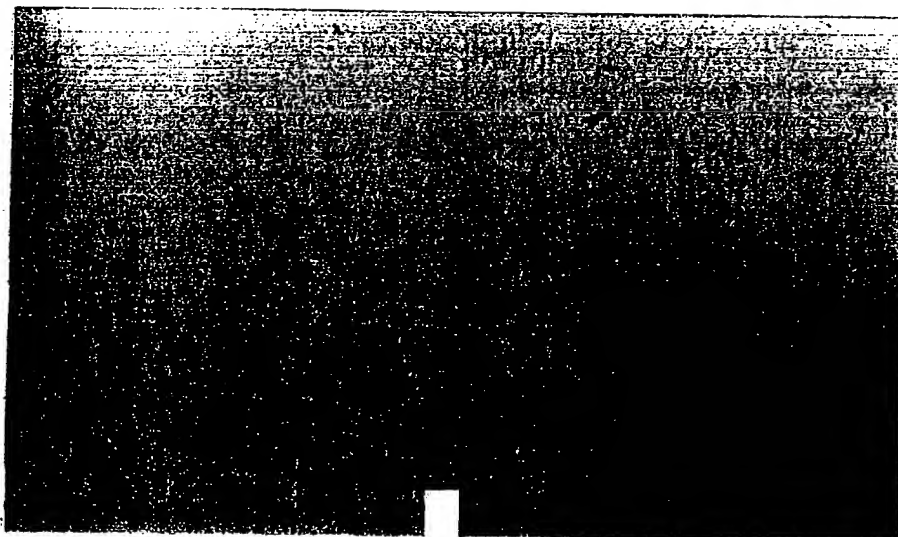


Fig. 7B

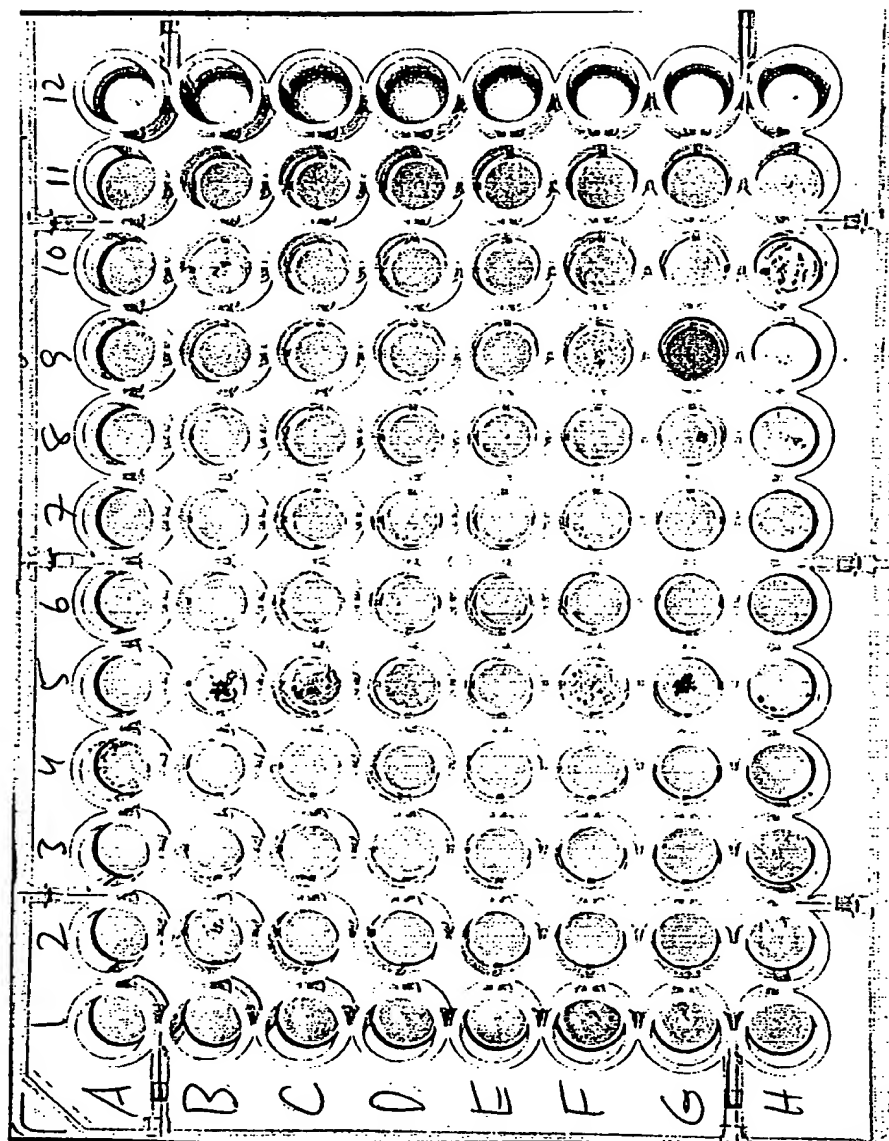


Fig. 8

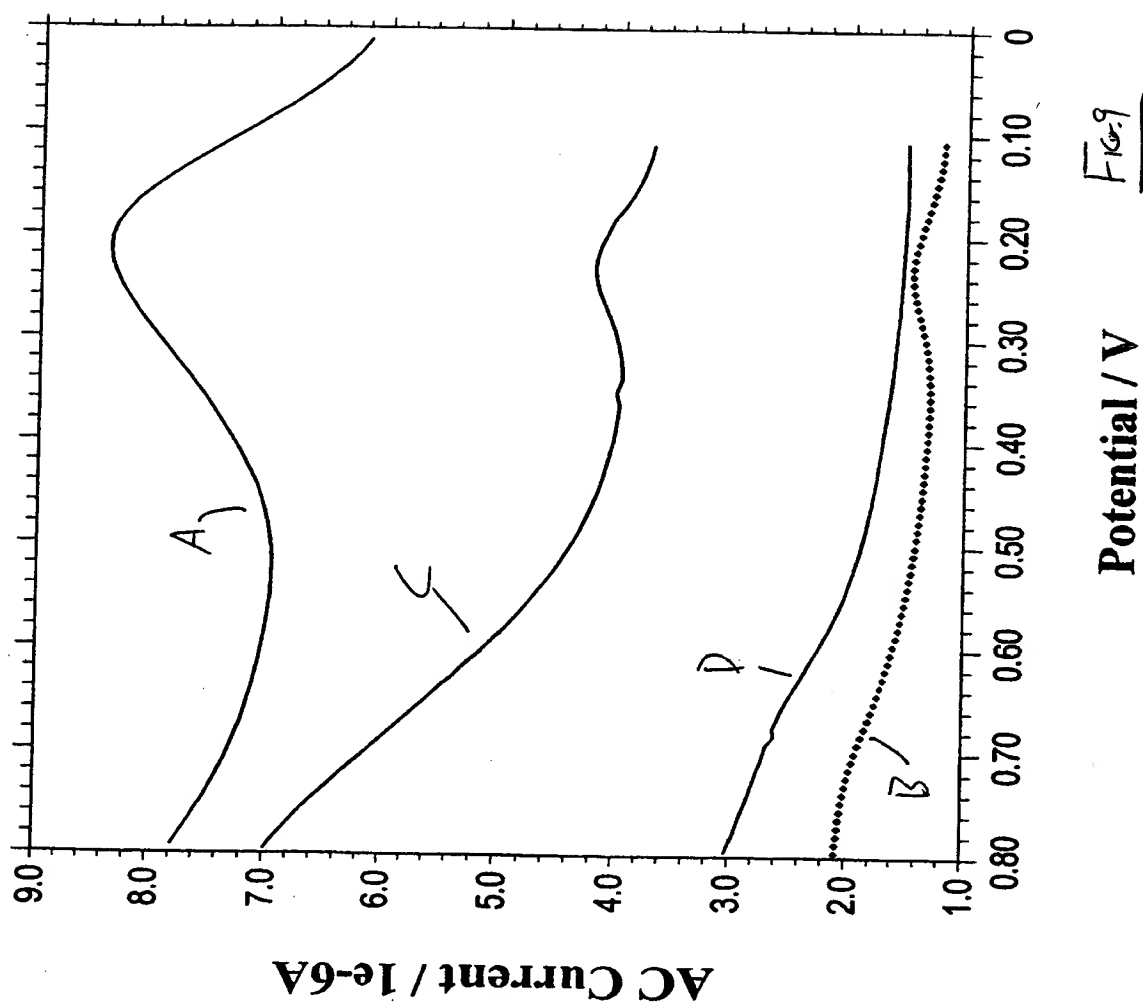


Fig. 9

Potential / V



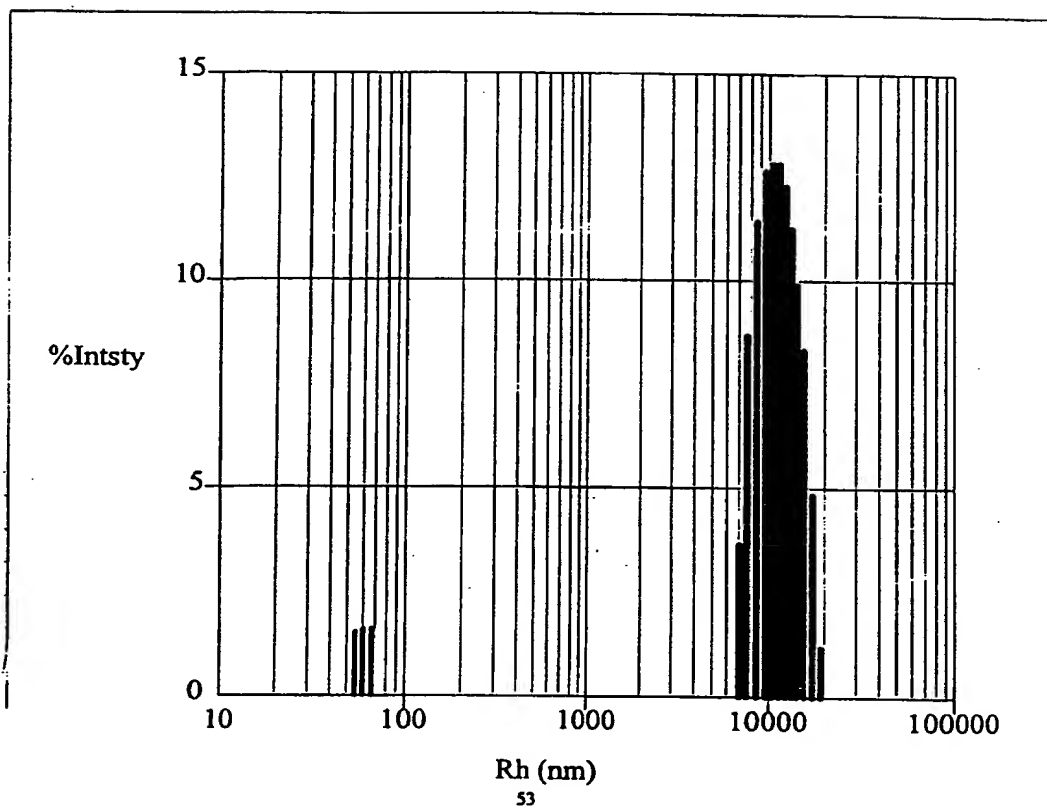
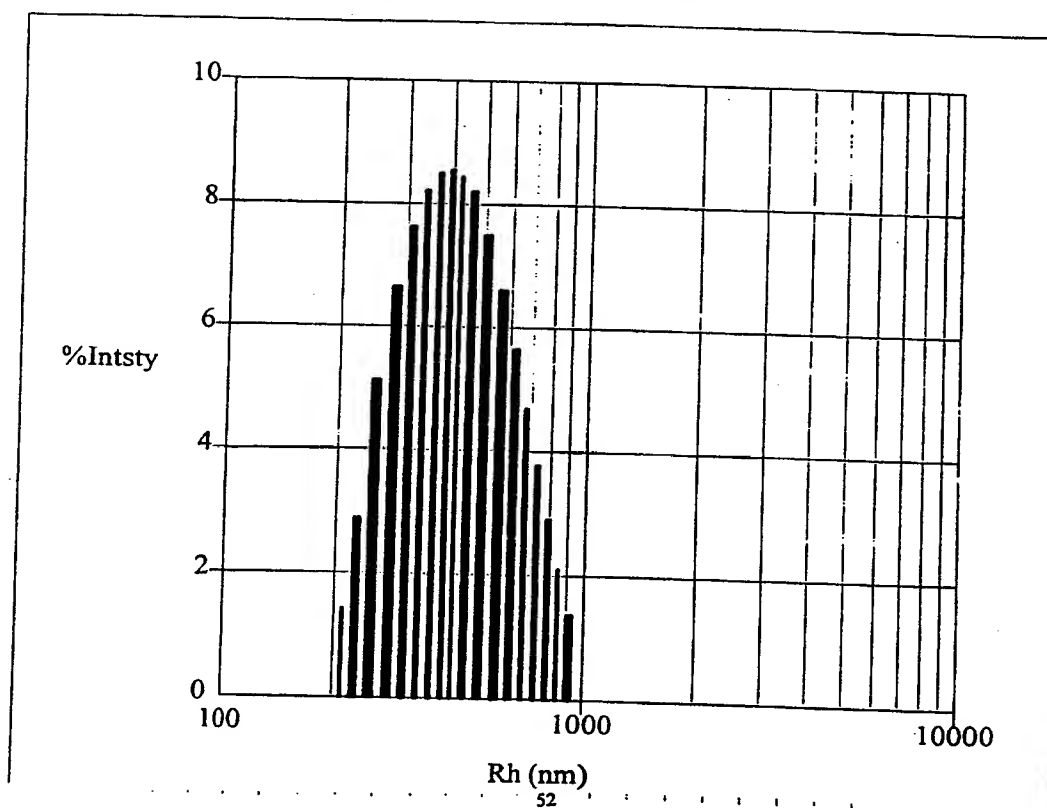
FIG. 10Regularization Histogram

Fig. 11Regularization Histogram

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B01J 19/00
- (21) International Application Number: PCT/US00/10171 (74) Agent: SHERWOOD, Pamela, J.; Bozicevic, Field & Francis LLP, 200 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).
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- (71) Applicant (*for all designated States except US*): THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY [US/US]; 900 Welch Road, Suite 350, Palo Alto, CA 94304 (US). (88) Date of publication of the international search report: 8 March 2001
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): BROWN, Patrick [US/US]; 76 Peter Courtis Circle, Stanford, CA 94305
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 00/63701 A3

(54) Title: MICROARRAYS OF POLYPEPTIDES

(57) Abstract: Microarrays of polypeptides on a solid support are provided. The microarray compositions find use in the multiplexed detection and quantitation of ligands, e.g. antigens or antibodies, in a miniaturized format. The substrate is used for detecting binding of ligands to a plurality of polypeptides for screening and diagnostic purposes.

# INTERNATIONAL SEARCH REPORT

International Application No  
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**A. CLASSIFICATION OF SUBJECT MATTER**  
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**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 G01N B01J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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X	WO 97 42507 A (ISIS INNOVATION LIMITED) 13 November 1997 (1997-11-13) abstract page 3, line 15 -page 4, line 16 page 7, line 18 - line 25 page 9, line 14 -page 10, line 3 page 4, line 14 - line 26 ----- -/--	1-12

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

8. Claims: 161,163 (when dependent upon claim 159)

Method comprising the formation of a SAM on a colloid surface.

9. Claims: 162,163 (when dependent upon claim 159)

Method comprising the formation of a SAM using a carboxylate.

10. Claim : 170

Method comprising the provision of at least two binding species capable of binding a neurodegenerative disease aggregate- or fibril-forming species forming a linker.

11. Claims: 171,172

Methods for drug screening, comprising the exposure of cells/solutions expressing/comprising a neurodegenerative disease aggregate-or fibril-forming species to a drug.

12. Claim : 173

Method comprising introducing energy in a fluid.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/01997

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60/155,937 24 September 1999 (24.09.1999) US

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Church Street, Newton, MA 02458 (US).

(74) Agent: OYER, Timothy, J.; Wolf, Greenfield & Sacks,  
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IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,  
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(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU,  
MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM,  
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26 July 2001

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: RAPID AND SENSITIVE DETECTION OF ABERRANT PROTEIN AGGREGATION IN NEURODEGENERATIVE DISEASES

(57) Abstract: Methods, assays, and components are described in which biological samples can be rapidly and sensitively analyzed for the presence of species associated with neurodegenerative disease. Techniques and components are provided for diagnosis of disease, as well as for screening of candidate drugs for treatment of neurodegenerative disease. The techniques are simple, extremely sensitive, and utilize readily-available components. Binding species, capable of binding a neurodegenerative disease aggregate-forming or fibril-forming species, are fastened to surfaces of electrodes and surfaces of particles, or provided free in solution, to bind fibril-forming species and/or be involved in aggregation.

# INTERNATIONAL SEARCH REPORT

International Application No

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**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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Y	page 6, line 16-20 page 15, line 6-24 page 18, line 24 -page 19, line 3 page 33, line 20 -page 35, line 6 claims 40-46; figure 16; example 17 --- -/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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16.02.01

Name and mailing address of the ISA

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Goetz, M



## INTERNATIONAL SEARCH REPORT

Inte. l. Application No

PCT/US 00/01997

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	page 7, line 20 -page 8, line 15 claims 1-9; examples 1,2 ---	2
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Y	page 8, line 1-7 page 14, line 15-32 page 15, line 13-49 claims 14-17; examples 9-12 ---	2
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Y	page 5, line 13-15 page 5, line 38-40 page 10, line 10-25 claims 1,25-28; examples 1.4,1.5 ---	2
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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/01997

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 00/01997

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 00/01997

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 14-24, 28, 53-56, 59, 152, 174-180  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:  
1-13, 25-52, 57, 58, 60-128, 131, 132, 135-151, 153-164, 170-172
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 14-24, 28,53-56, 59, 152, 174-180

A. Claims 14-24,28,53-56,59,152 and 174-180:

These claims relate to an extremely large number of possible embodiments. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the articles, methods, kits, systems or compositions claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Claim 14 is unclear (Art.6 PCT), as it relates to a binding species, which however cannot be found in e.g. claims 6 and 7.

Claim 24 is unclear (Art.6 PCT), as it generically relates to "fusion" which can however only be found in claims 15 and 16.

Claim 152 is unclear (Art.6 PCT), as it relates to a signaling entity, which however cannot be found in many of the previous claims.

Claim 174 is unclear (Art.6 PCT), as it relates to "A method as in any preceding claim"; however, only some of the previous claims relate to such method.

Claims 175-177 are unclear (Art.6 PCT), as they relate to an article, which however cannot be found in e.g. claims 2 and 71-74.

Claims 179 and 180 are unclear (Art.6 PCT), as they relate to a sample, which however cannot be found in e.g. claims 1-8.

Additionally, in view of the non-unity objections raised, it is impossible to attribute the subject-matter claimed in present claims 14-24,28,53-56,59,152 and 174-180 to a unique one of the 14 inventions found in the set of claims. Consequently, as the determination of their exact scope is rendered impossible, no search has been carried out for claims 14-24,28,53-56,59,152 and 174-180.

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The following deficiencies have also been noted:

B. Claims 25-37 and 60-70:

Claims 25-37 and 60-70 are unclear (Art.6 PCT), as they relate to "A kit as in claims 1 - 5", which kit however cannot be found in claims 2 and 3 which relate to a composition and an article.

Claims 25-27,60 and 63-70 are unclear (Art.6 PCT), as they relate to an article, which however cannot be found in e.g. claim 2.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims 31-37 and 62 are unclear (Art.6 PCT), as they relate to a surface, which however cannot be found in e.g. claim 2.

Claims 63 and 65-70 are unclear (Art.6 PCT), as their preamble refers to "An article as in claim ..."; however, the claims to which it is referred recite a kit, not an article.

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C. Claims 38-52:

Claims 38-52 are unclear (Art.6 PCT), as they relate to "A kit as in claims 3-5", which kit however cannot be found in claim 3 which relates to an article.

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D. Claims 128,131,132 and 136:

These dependent claims do comprise wrong references. As far as could be understood, claim 128 should be dependent upon claim 127, claim 131 upon claim 9, claim 132 upon claim 131, and claim 136 upon claim 9.

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E. Claim 138:

Claim 138 is unclear (Art.6 PC), as it relates to particles and/or magnetic beads, which have not been mentioned in any of the previous claims. As far as could be understood, claim 138 should be dependent upon claim 123.

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For the purpose of assigning the claims mentioned in paragraphs B. - E. above to one of the groups of invention, it was therefore assumed that they either directly or indirectly (that is, via another dependent claim) relate to the kit of claim 1 or method of claim 9; hence, the said claims were assigned to the first invention.

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The Applicant is notified that the claim numbering does not comply with Rule 6.1 b) PCT, as some claim numbers are missing and the use of claim numbers such as "80.1" is not allowed.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1,3,4,5,9,10,13,25-52,60-70,75-128,131,132,135-151,  
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Articles having surfaces and binding species capable of binding a neurodegenerative disease aggregate- or fibril-forming species, kits comprising the said article, as well as methods using the said articles.

2. Claim : 2

Composition comprising a binding species capable of binding a neurodegenerative disease aggregate- or fibril-forming species and an electronic signaling entity.

3. Claims: 6,7,71-74

System comprising a neurodegenerative disease fibril or aggregate and at least two particles.

4. Claims: 8,57,58

Composition comprising a binding species capable of binding a neurodegenerative disease aggregate- or fibril-forming species fastened to a moiety that can coordinate a metal.

5. Claims: 11,12,153-157

Method involving binding species which are not neurodegenerative disease aggregate- or fibril-forming species, said method involving the conversion into neurodegenerative disease aggregate- or fibril-forming species.

6. Claims: 159,163 (when dependent upon claim 159),164

Method comprising the formation of a SAM using a surfactant.

7. Claims: 160,163 (when dependent upon claim 159)

Method comprising the formation of a SAM using a carboxylate.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.